

**ACUTE AND CHRONIC INDIVIDUALISED  
PSYCHOPHYSIOLOGICAL STRESS ASSESSMENT  
OF ELITE ATHLETES THROUGH NON-INVASIVE  
BIOCHEMICAL ANALYSIS**

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A thesis submitted

in partial fulfilment of the requirements for the Degree of

Doctor of Philosophy

In Biochemistry

School of Biological Sciences

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New Zealand

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## Abstract

Intense exercise is known to cause alterations in the psychophysiological status of an athlete. Monitoring the health and recovery of an athlete is imperative for the maintenance of performance and reduced fatigue and injury incidence. The physicality associated with select sports results in significant elevations and suppression of psychophysiological biomarkers that are often modulated by game-related impacts, intense training regimes and psychosocial aspects associated with the professional era. The aim of the studies outlined in this thesis were to determine the effectiveness of selected “stress” markers in several sports that result in significant “stress”, and quantify the level of acute and chronic “stress” following individual games and competitions to improve athlete management and recovery.

Study one aimed at developing a new strong-cation exchange high performance liquid chromatography (SCX-HPLC) method for the detection and quantification of urinary pterins and creatinine in a body-building cohort completing high intensity resistance training. The method had an intra- and inter-assay variability of 3.04 % and 5.42 % respectively, with visibly clear peaks and no tailing. Urinary neopterin (NP) and 7,8-dihydroneopterin during a week of competitive natural body-building did not significantly change indicating no alteration in immune system function and oxidative stress. It did provide evidence for the use of specific gravity as a similarly reliable method for urine volume correction following exercise.

Study two focused on a playoff game of elite amateur rugby. The time course changes of NP, cortisol, salivary immunoglobulin A (sIgA) and myoglobin in 11 elite amateur rugby players were measured up to 86 hours post-game. Cortisol increased 4-fold, myoglobin 2.85-fold, NP 1.75-fold and total NP 2.3-fold, all significant, whilst sIgA did not change. All markers returned to baseline within 17 hours providing valuable information for sample collection schedule optimization. Respiratory elastance was also measured by ventilation for the assessment of exercise induced lung inflammation/injury following the game (Chapter three). There was an increase in elastance in selected individuals that did not correlate with either global positioning system (GPS) or impact data. It was shown however, that a ventilator is capable of measuring respiratory changes in a conscious and healthy individual.

Study three focused on the final three games of professional rugby in the 2013 ITM Cup. The acute and cumulative changes in the same four markers were analysed following three home games. There were significant increases in NP, total NP, cortisol and myoglobin along with significant suppression of sIgA ( $p < 0.05$ ). Large intra- and inter-individual variation existed between players with changes associated with total impacts. Moreover, impact induced muscle damage may account for changes in oxidative status. Specific gravity (SG) was shown to be a more reliable marker for urine volume correction in comparison to creatinine; while some players showed signs of cumulative stress.

Study four examined stress in a professional team throughout the 22 week 2014 Super 15 competition. Part one investigated changes in oxidative stress and muscle damage markers to solidify the muscle damage/oxidative status theory postulated in the previous study. Experimental evidence showed iron and myoglobin are separately capable of oxidizing 7,8-dihydroneopterin to NP *in vitro*. It was then identified that players who suffered the greatest muscle damage as a result of impacts also had the greatest change in oxidative status (NP). This evidence suggests rugby union induces significant alterations in oxidative status that may be exacerbated by the impact induced release of myoglobin.

Part two measured urinary NT-proBNP during the last two consecutive home games to identify whether rugby union causes significant cardiovascular stress and if the pre to post-game change can be explained by GPS technology. Significant individualized elevations were observed in games one and two which did not correlate with any GPS measurements or impacts. Concentrations returned to normal ~ 36 hours post-game suggesting no permanent damage to cardiac muscle had occurred. The lack of correlation suggests GPS technology is not an accurate measure of cardiovascular stress in professional rugby union.

Part three involved the measurement of cortisol, total NP and sIgA throughout the season to assess the degree of cumulative stress. Samples were taken at regular intervals ~ 36 hours post-game for 22 weeks. Extreme inter-individual variation was present. Select individuals showed continual elevation in immune system activation and psychophysiological stress, whilst others presented with a continual decline in immune system function. Collectively however, minor deviations from baseline in all markers were observed and participation in long distance travel did not significantly affect the psychophysiological status of the group.

Together this suggests a season does not cause an accumulation in psychophysiological stress, although careful individual player analysis is warranted.

Understanding rugby union positional demands is essential for training program specification and position specific development of players. Part four used GPS, video-analysis and biochemical analysis to identify positional demands in five regular season games. Forwards tended to be involved in more impacts and covered less distance, while backs covered more distance and carried the ball into contact more regularly. There was no difference in the psychophysiological status between positions indicating both aspects of stress (impacts and distance covered) may induce a similar response. Alternatively, individual biological variation may be solely responsible for this change suggesting careful consideration should be given when using traditional work-load measures such as GPS when quantifying “stress”.

Part five assessed the effectiveness of varied recovery interventions. Total NP, cortisol, myoglobin and sIgA were measured pre- post- and ~ 36 hours post game to identify which intervention was most effective at returning players to a psychophysiological state that allowed for the resumption of normal training. Findings concluded the immediate post-game strategy employed by the team (cold bath, consumption of protein and carbohydrates, compression garments and eight hours sleep) seemed to provide the greatest psychophysiological improvement regardless of the “next-day” intervention. There was large inter-individual variation and players were still in a state of recovery ~ 36 hours post-game as indicated by the elevated total NP and sIgA concentrations.

Study five had four aspects. Develop a new, cost-effective and simple reverse phase HPLC (RP-HPLC) method for the quantification of urinary myoglobin in a clinically relevant range, quantify the level of structural stress following a simulated mixed martial arts (MMA) contest, determine whether cold water immersion attenuates the level of inflammation and muscle damage following a contest, and whether this hypothesized attenuation may be explained by cryotherapy induced mononuclear cell activation suppression *in vitro*. The RP-HPLC method had an intra- and inter-assay variations from 0.32 - 2.94 %. Linearity was in the range of 5 – 1000 µg/mL which detected significant increases in urinary myoglobin following the MMA contest. Total NP was found to significantly increase following the contest and return to approximately pre-contest levels 24 hours later for the passive group only. Cold water immersion was further found to attenuate the total NP increase in the first

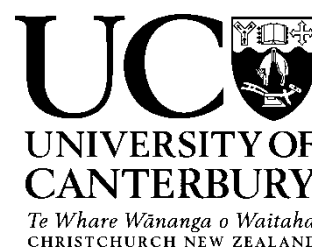
two hours post-contest solidifying its use as a recovery technique following intense exercise, while cryotherapy significantly suppressed T-cell activation. This study provides a reliable and repeatable assay for muscle damage quantification in a clinically relevant range, evidence of the physicality associated with MMA, and indicates cold water immersion is a reliable recovery intervention that may impart its positive benefits through T-cell suppression.

The data generated by these investigations highlights the necessity for individual physiological analysis. Group data often masks the extreme variation that exists in clinical and exercise trials where treatment and management of athletes is conducted for recovery and performance. Biochemical analysis provides an added sophistication of work-load and psychophysiological assessment that common technological methods cannot emulate. With a lack of correlation between the quantitative changes in specific non-overlapping biomarkers and GPS, video-analysis and questionnaires, it would seem pertinent to develop a non-invasive quantitative approach in elite sport to understand the level of exercise-induced psychophysiological stress for the precise management of athletes.



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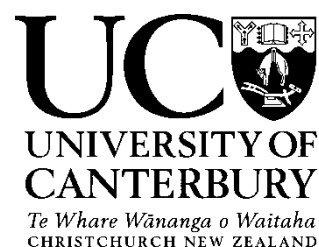
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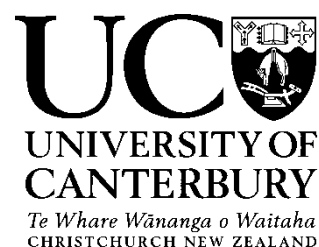
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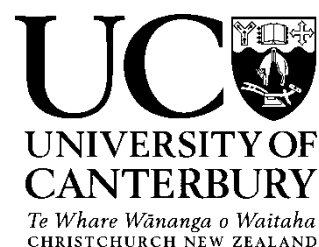
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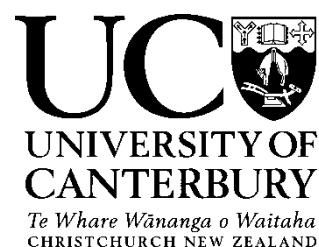
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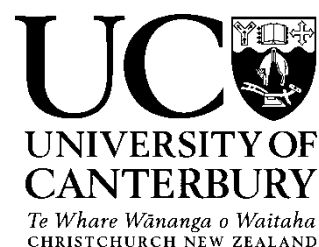
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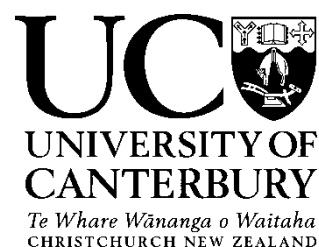
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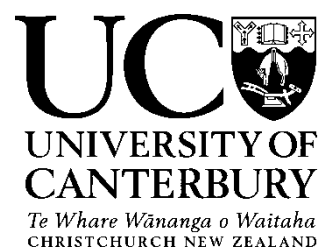
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**Positional demands of professional rugby**

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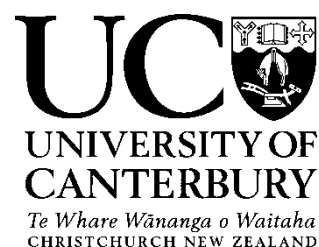
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**Effect of varied recovery interventions on markers of psychophysiological stress in professional rugby union**

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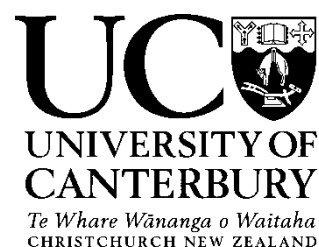
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**Measurement of urinary myoglobin by RP-HPLC and effective sample stabilisation by alkalis**

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**The candidate completed the ethics application, responsible for the recruitment of all subjects, completed half the method development, completed all statistical analysis and wrote the manuscript.**

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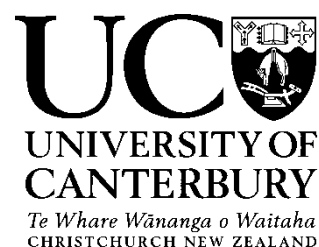
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**Effect of cold water immersion on the physiological stress response following a mixed martial arts contest: Effect of cryotherapy on blood derived mononuclear cell activation**

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**The candidate completed the ethics application, responsible for the recruitment of all subjects, completed half of all sample collection and sample analysis, completed all statistical analysis and wrote the manuscript.**

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## Publications

Lindsay, A., Janmale, T., Draper, N., and Gieseg, S. P. (2014). "Measurement of changes in urinary neopterin and total neopterin in body builders using SCX HPLC." Pteridines **25**(2): 53-63.

Lindsay, A., Lewis, J., Scarrott, C., Draper, N., and Gieseg, S. P. (2014). "Changes in acute biochemical markers of inflammatory and structural stress in rugby union." Journal of Sports Sciences **33**(9): 882-891.

Lindsay, A., Chiew, Y., Bernard, A., Davidson, S. M., Redmond, D., Pretty, C., Chase, G. J., Shaw, G. M., Gieseg, S. P., and Draper, N. (2014). "Lung injury and respiratory mechanics in rugby union." Journal of Sports Medicine and Physical Fitness. "In Press".

Lindsay, A., Lewis, J., Scarrott, C., Gill, N., Gieseg, S. P., and Draper, N. (2015). "Assessing the effectiveness of selected biomarkers in the acute and cumulative physiological stress response in professional rugby union through non-invasive assessment." International Journal of Sports Medicine. doi: 10.1055/s-0034-1398528.

Lindsay, A., Healy, J., Mills, W., Lewis, J., Gill, N., Draper, N., and Gieseg, S. P. (2015). "Impact induced muscle damage and urinary pterins in professional rugby: 7,8-dihydroneopterin oxidation by myoglobin." Scandinavian Journal of Medicine and Science in Sports. doi: 10.1111/sms.12436.

Lindsay, A., Lewis, J., Gill, N., Draper, N., and Giese, S. P. (2015). "Global positioning system technology does not correlate with cardiovascular stress measured as urinary NT-proBNP in professional rugby union." International Journal of Sports Physiology and Performance. "In Review".

Lindsay, A., Lewis, J., Gill, N., Giese, S. P., and Draper, N. (2015). "Immunity, inflammatory, and psychophysiological stress response during a competition of professional rugby union." European Journal of Sport Science. "In Review".

Lindsay, A., Draper, N., Lewis, J., Giese, S. P., and Gill, N. (2015). "Positional demands of professional rugby". European Journal of Sport Science. Doi: 10.1080/17461391.2015.1025858

Lindsay, A., Lewis, J., Gill, N., Giese, S. P., and Draper, N. (2015). "Effect of varied recovery interventions on markers of psychophysiological stress in professional rugby union". European Journal of Sport Science. Doi: 10.1080/17461391.2015.1029982

Lindsay, A., Carr, S., Draper, N., and Giese, S. P. (2015). "Urinary myoglobin quantification by HPLC; an alternative measurement for exercise-induced muscle damage." Analytical Biochemistry: Methods in the Biological Sciences. "In Review".

Lindsay, A., Carr, S., Othman, M. I., Davies, S., Marks, E., Petersen, C., Draper, N., and Giese, S. P. (2015). "Effect of cryotherapy on the physiological stress response and blood derived mononuclear cell activation following a mixed martial arts contest: a pilot study." Pteridines. "In Review".

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\*  $p < 0.05$ .

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## List of Equations

1)  $\sqrt{((a_{y1} - a_{y-1})^2 + (a_{x1} - a_{x-1})^2 + (a_{z1} - a_{z-1})^2)}$

GPS calculation of player-load where  $a_y$  = anteroposterior acceleration,  $a_x$  = mediolateral acceleration and  $a_z$  = vertical acceleration.

2)  $\text{Concentration}_{\text{SG normalized}} = \text{Concentration}_{\text{specimen}} \cdot (\text{SG}_{\text{reference}} - 1) / (\text{SG}_{\text{specimen}} - 1)$

Equation used for the correction of a urine volume for a specific analyte.

3)  $\%CV = (\text{SD}_{\text{samples}} / \text{Mean}_{\text{samples}}) \times 100$

Calculation of the intra- and inter-assay variation of an analytical technique for assessment of reliability and repeatability.

4)  $P_{aw}(t) = R_{rs}Q(t) + E_{rs}V(t) + P_0$

A measure of a player's respiratory mechanics where  $P_{aw}(t)$  is airway pressure,  $t$  is the time,  $R_{rs}$  is the airway resistance,  $Q(t)$  is the flow,  $E_{rs}$  is the respiratory elastance,  $V(t)$  is the air volume entering the lung (tidal volume) and  $P_0$  is the offset pressure.

5)  $E_{drs}(t) = (P_{aw}(t) - R_{rs}Q(t) + P_0) / V(t)$

Measurement of a player's time varying elastance.

6)  $\text{Total Lung Capacity} = 7.99 * \text{Height} - 7.08$

Equation used to estimate a person's lung capacity

## Abbreviations

ACN	Acetonitrile
ACTH	Adrenocorticotrophic hormone
AFL	Australian Rules Football
AmPO <sub>4</sub>	Ammonium phosphate
ANS	Autonomic nervous system
ANOVA	Analysis of variance
ARDS	Acute respiratory diseases
ARF	Acute renal failure
AUCE <sub>drs</sub>	Area under the curve for respiratory elastance calculation
AVP	Arginine-vasopressin
a <sub>x</sub>	Mediolateral acceleration
a <sub>y</sub>	Anteroposterior acceleration
a <sub>z</sub>	Vertical acceleration
BH <sub>2</sub>	7,8-dihydrobiopterin
BH <sub>4</sub>	Tetrahydrobiopterin
BNP	Brain natriuretic peptide
BP	Biopterin
(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub> O	Diethylether
C <sub>2</sub> H <sub>3</sub> O <sub>2</sub> .NH <sub>4</sub>	Ammonium acetate
CAIII	Carbonic anhydrase III
CH <sub>2</sub> Cl <sub>2</sub>	Dichloromethane
CK	Creatine kinase
CK-BB	Creatine kinase-brain
CO <sub>2</sub>	Carbon dioxide
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein

CSF	Cerebrospinal fluid
CT	Computerized tomography
CV	Co-efficient of variation
cTnI	Cardiac troponin I
cTnT	Cardiac troponin T
DALDA	Daily analyses of life demands of athletes
DNA	Deoxyribonucleic acid
DOMS	Delayed onset muscle soreness
ECG	Electrocardiography
$E_{drs}$	Dynamic lung elastance
$E_{rs}$	Respiratory elastance
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ERS	European Respiratory Society
$Fe^{2+}/Fe^{3+}$	Ferrous/Feric iron
$Fe^{3+}/Fe^{4+}$	Feric/Ferryl iron
FO	Functional overreaching
GFAP	Glial fibrillary acidic protein
GPS	Global positioning system
GREs	Glucocorticoid responsive elements
GTP	Guanosine tri-phosphate
GTPCH-1	Guanosine tri-phosphate cyclohydrolase 1
H <sub>2</sub> O	Water
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HCL	Hydrochloric acid
HOCL	Hypochlorous acid
HPA	Hypothalamic-pituitary axis
HPLC	High performance liquid chromatography

I <sub>2</sub>	Iodine
I <sup>-</sup>	Iodide
ICU	Intensive care unit
Ig	Immunoglobulin
IL-1β	Interleukin-1β
IL-1ra	Interleukin-1ra
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-15	Interleukin-15
IFN-γ	Interferon-γ
IRB	International Rugby Board
KI	Potassium iodide
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
MMA	Mixed martial arts
MRI	Magnetic resonance imaging
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
Na <sub>2</sub> H <sub>2</sub> PO <sub>4</sub>	Sodium dihydrogen orthophosphate
NFL	National Football League
NFO	Non-functional overreaching
NHL	National Hockey League
NIH	National Institute of Health
NK	Natural killer
NO	Nitric oxide
NP	Neopterin

NSE	Neuron specific enolase
NT-proBNP	N-terminal prohormone of brain natriuretic peptide
O <sub>2</sub>	Oxygen
OTS	Over-training syndrome
oxLDL	Oxidized low-density lipoprotein
$P_{aw}(t)$	Airway pressure
PBS	Phosphate buffered saline
PEEP	Positive end-expiratory pressure
PGC-1 $\alpha$	Peroxisome proliferator-activated receptor gamma coactivator-1 $\alpha$
PMA	Phorbol myristate acetate
$P_o$	Offset pressure of ventilator
PPI	Present pain intensity
PTSD	Post-traumatic stress disorder
$Q(t)$	Air flow
RIA	Radioimmunoassay
ROS	Reactive oxygen species
RP	Reverse phase
$R_{rs}$	Airway resistance
S100B	S100 calcium binding protein B
SAM	Sympathetic adrenal-medullary axis
SCX	Strong-cation exchange
SD	Standard deviation
SEM	Standard error of the mean
SF-MPQ	Short form McGill pain questionnaire
SG	Specific gravity
sICAM-1	Soluble intercellular adhesion molecule-1
sIgA	Salivary immunoglobulin A
SIMV	Synchronized intermittent mandatory ventilation

SOD	Superoxide dismutase
SPONT	Spontaneous
TBARS	Thiobarbituric acid reactive substances
TBI	Traumatic brain injury
TNF- $\alpha$	Tumor necrosis factor-alpha
UCH-L1	Ubiquitin carboxyl terminal hydrolase-L1
URTI	Upper respiratory tract infection
UV	Ultraviolet
$V(t)$	Tidal volume
VAS	Visual analogue scale
W	Watts
WADA	World anti-doping agency
WBC	Whole body cryotherapy
WHO	World Health Organization
WOB	Work of breathing
XP	Xanthopterin

# Chapter 1

## Introduction

### 1.1 OVERVIEW

Biomarkers of physiological and psychological stress are widely used in a sporting and clinical context to provide a quantitative assessment of an athlete or patients health. The information gathered can be utilised to monitor and manage individual recovery from exercise, illness and disease progression or severity. Often professional athlete's recovery and performance are measured through qualitative and subjective analyses. Global positioning system (GPS) and video analysis are current technologies utilised to gauge the workload of an athlete, while questionnaires provide subjective feedback allowing medical and training staff to alter and implement individual strategies. This type of approach is considered robust, non-invasive and reasonably inexpensive, however it does not account for the degree of individual physiological variation. These observational approaches provide a quantitative valuation that is independent of several influential variables including personal evaluation of fatigue or soreness. Biochemical assessment through saliva and urine forgoes the problems associated with quantifying exercise stress in this routinely used subjective and group manner. It is imperative each individual athlete is monitored as their own entity due to the large inter-individual variation in physiological characteristics so often experienced in exercise research.

Professional rugby union offers an alternative avenue to analyse this individual variation. It is a sport that incorporates high intensity, high frequency and physical contact that has been shown to cause significant elevations in markers of psychophysiological stress. It should therefore provide the ideal opportunity to trial and present the assessment of individual biochemical variation. The literature examining biochemical changes in professional sport is extensive. Information of this nature does provide us with a comprehensive understanding of the physiological stresses involved in various sports. However the application based use of this research for the management of athlete health and performance is widely neglected. The research described in this thesis is conducted in a non-invasive and stress free manner to provide the group and individual psychophysiological assessment of professional rugby union stress as a surrogate to serious trauma. This information can be used to gauge whether the

development of an individualized “biological passport” through biochemical analysis could be implemented to facilitate monitoring of athlete recovery and health. The aim of this research was therefore to utilize the development of diagnostic techniques for patient monitoring and disease progression in common medical practices and trial them in a sport that resembles and encompasses serious trauma.

## **1.2 CONTACT SPORT**

The added demand of contact sport creates further psychophysiological stress that does not accompany single facet based aerobic or anaerobic sport. While endurance based and non-contact field sports activate inflammatory processes and cause changes in hormonal responses (Banfi *et al.*, 2010; Benoni *et al.*, 1995; Ekblom *et al.*, 2006; Gunga *et al.*, 2002; Ispirlidis *et al.*, 2008; Mousavi *et al.*, 2009; Sprenger *et al.*, 1992; Tharp 1991), high force collisions resulting in significant amounts of muscle damage accompanied by an aerobic or anaerobic component add a compounding effect. Extensive research has been conducted on the “collision” based sports that include rugby union, boxing, American football (NFL), ice hockey (NHL), Australian Rules Football (AFL) and rugby league (Brewer *et al.*, 2010; Coutts *et al.*, 2007; Graham *et al.*, 2011; Hoffman *et al.*, 2005; Schwellnus *et al.*, 2012; Selanne *et al.*, 2014). To evaluate the reliability and precision of the research to be undertaken, a sport that causes significant changes in both physiological and psychological profiles was required that was comparable to the distress experienced in trauma victims.

One of the most physically demanding field sport in the world (Mashiko *et al.*, 2004), rugby union is dominated by high force and frequency collisions coupled with a substantial anaerobic component interspersed with periods of lower intensity aerobic work (Coughlan *et al.*, 2011). This combination of stresses known to cause significant changes in selective biomarkers (Cunniffe *et al.*, 2010) provides a compelling argument for the use of rugby as an experimental sport for the development of a set of specific and non-overlapping markers of “stress”. Additionally, as New Zealand’s unofficial national sport that has participation numbers over 150,000 ([www.rugbyworldcup.com](http://www.rugbyworldcup.com)) in a nation of approximately 4.5 million people, the access to both amateur and professional players made rugby union the ideal sport for research of this nature.



#### 1.2.1 Rugby Union: The Game

Since its emergence in 1871, rugby union has developed world-wide popularity extending participation into 100 countries across five continents (Quarrie and Hopkins 2007). New Zealand in particular has been established as one of the dominant nations since its initial “originals” tour of Britain in 1905. The 2011 World Cup hosting and victory saw further development for passion of the game. A six percent increase in under-12’s participation and a seven percent increase in coaches ([www.rugbyworldcup.com](http://www.rugbyworldcup.com)) were observed as a result. The event was broadcast in 207 countries, including the USA for the first time, while more than 1.35 million fans attended the matches which included 133,000 traveling from abroad.

The New Zealand rugby season which is similar to that of other Southern Hemisphere playing nations, is comprised of provincial, franchise and international competition of approximately 40 weeks a year of play.

- Super 15 – February to July
- The Rugby Championship/Bledisloe Cup – August to October
- ITM Cup (provincial competition) – August to October
- European Tour - November

The New Zealand Super 15 franchises that compete against similar opposition from Australia and South Africa are comprised of 14 provincial teams. Only a select number of players are offered contracts to the five franchises (Canterbury Crusaders, Otago Highlanders, Wellington Hurricanes, Waikato Chiefs and Auckland Blues), while the remaining compete in elite level club competition. However, not all professional New Zealand players compete in national competitions, with some accepting lucrative offers from clubs in Japan and Europe.

#### 1.2.2 Characteristics, Demands and Trends in Rugby Union

Rugby union is played with 15 players a side for two periods of 40 minutes separated by a 10 minute break. The ball is in play for approximately 30 - 35 minutes (Quarrie and Hopkins 2007) which in comparison to other contact sports (American football - 11 minutes) might be the confounding factor for the higher injury incidence rates observed in rugby (Brooks *et al.*, 2005). There are two general types of positions within the 15-man game; forwards (No. 1 -

8) and backs (No. 9 - 15). Each position within a team has a specific purpose with selection criteria based on the ability of a player to perform the required tasks. While backs are typically smaller in size ( $95.7 \pm 2.3$  kg) compared to their forward counterparts ( $111.1 \pm 2.9$  kg) (Quarrie and Hopkins 2007), their roles require more agility and speed for activities such as ball carrying and gaining ground which is represented by less time standing still (Quarrie *et al.*, 2013). Forwards however, require large amounts of strength and power to deal with the forces in specific tasks such as scrums, lineouts and securing possession (Quarrie and Wilson 2000; Reilly 1997). Scrums in particular induce forces greater than 16.5 kN (Preatoni *et al.*, 2012) that unfortunately account for 40 % of all catastrophic rugby injuries, most of which involve the spinal cord (Quarrie *et al.*, 2002).

Since the advent of the professional era (1995), more time has been dedicated to the physical preparation, skill development and tactical aspects of players with  $\dot{V}O_2\text{max}$  values in the region of 45 - 55  $\text{ml.kg}^{-1}.\text{min}^{-1}$  (Duthie *et al.*, 2003). This change has also lead to the improvement of monitoring technologies used to quantify the level of stress a player is subjected to during training and competition. Previous stress assessment has utilized movement analysis (Deutsch *et al.*, 1998; Docherty *et al.*, 1988; Roberts *et al.*, 2008), heart rate monitoring and lactate profiling (Deutsch *et al.*, 1998; McLean 1992). In the modern era, GPS and video-analysis technologies have been developed by companies that supersede this technology that are used in today's game to manage a player's recovery and workload.

Global positioning system technology has calculated players covering anywhere from 5000 – 7000 m per game (Austin *et al.*, 2011; Cunniffe *et al.*, 2009) that is comprised of 37 % walking, 27 % jogging, 14 % striding, 5 % high intensity running and 6 % sprinting (Cunniffe *et al.*, 2009). Ninety-five percent of the game activities are normally 30 seconds or less (Duthie *et al.*, 2003) with forwards spending a greater amount of time at high intensity compared to backs (12 – 13 % vs. 4 – 5 %); most likely a result of their close proximity to the action. Backs however, tend to complete more sprints and spend greater time at higher running speeds (Cunniffe *et al.*, 2009; Deutsch *et al.*, 2007).

In addition to aerobic and anaerobic components, video-analysis data and laboratory based experiments have shown the number of tackles per hour within rugby union can range from 142 – 166 (Fuller *et al.*, 2007; McIntosh *et al.*, 2010; Quarrie and Hopkins 2008) with forces exceeding 1000 N (Pain *et al.*, 2008) or 1.95 – 2.31 x bodyweight (Usman *et al.*, 2011).

When combined with tackle velocities ranging from  $3.1 \pm 1.2$  to  $7.1 \pm 3.5 \text{ m.s}^{-1}$  (Hendricks *et al.*, 2012), rugby union is certainly considered one of the most physically demanding sports.

In conjunction with technology changes, evolution of player demands has substantially transformed as well. The game has become faster and more physically demanding as a result of law alterations (“use it or lose it” law) that keep the game more attractive for audiences (Duthie *et al.*, 2003; Quarrie and Hopkins 2007). Both rucks and tackles have significantly increased since the onset of professionalism (Eaves and Hughes 2003; Quarrie and Hopkins 2007). This has increased the frequency of other game related activities such as lineouts, number of possessions, kicks in play (Eaves *et al.*, 2005) and decreased work to rest ratios from 1 : 20 in 2005 (Duthie *et al.*, 2005) to 1 : 5 in 2011 (Austin *et al.*, 2011). Player stature and mean body mass have also increased across all positions, (Fuller *et al.*, 2012; Quarrie and Hopkins 2007) which compensates for the change in positional play requirements and physicality of contact. Since 1975, there has been a decrease in the ectomorphic and endomorphic body types of players and a higher prevalence of mesomorphic physical appearances that also correlates with team success (Olds 2001). As a consequence of the increased number of tackles over time, injury prevalence has also become elevated to 9.84 injuries per 1000 athletic exposures where one exposure represents one player participating in one game (Usman and McIntosh 2013). The highest incidence rate is reserved for backs who are required to tackle opposition players at top speed causing the greatest number of injuries (shoulder) (Sundaram *et al.*, 2011). There is a strong correlation between injury and number of tackles or ruck events (Eaves and Hughes 2003), while up to 58 % of all game-related injuries are accounted for by tackles (Bathgate *et al.*, 2002; Bird *et al.*, 1998; Brooks *et al.*, 2005).

## 1.2 EXERCISE STRESS

Stress can be described as the state of the organism under the influence of external or internal forces, or “stressors”, which threaten to alter its dynamic equilibrium or homeostasis. The adaptive changes occurring in response to stressors can be both behavioural and physical (Mastorakos *et al.*, 2005), while a cascade of physiological responses ensues that acts to rectify or balance the altered state of the affected organism. Exercise initiates acceleration in cardiac output, respiration, and catabolism which redirects blood flow to provide the highest

perfusion and fuel to the aroused brain, heart and muscles (Chrousos and Gold 1992). These processes stimulate activation of the hypothalamic-pituitary (HPA) and sympathetic adrenal-medullary axes (SAM) which influence the brain and all body-organs during exposure to threatening stimuli (Gold *et al.*, 1988). Communication between immune and inflammatory processes and the central nervous system are well understood (Flier *et al.*, 1995), which explains the widespread research observing changes in immune system, inflammatory, endocrine and nervous system parameters. (For a detailed review of changes in inflammatory, immune, nervous and endocrine following strenuous exercise see (Pedersen and Hoffman-Goetz 2000)). As a result of rugby union's physical demands and relative homeostatic change in response to the game stress, it represents a stable model for assessing the effects of strenuous exercise on changes in psychophysiological stress.

### 1.2.1 Rugby Union Induced Stress

Rugby union has been analysed extensively for changes in both physiological and psychological parameters in an acute and chronic context. Attributed to the high force impacts and intermittent nature of the game, acute changes in several biomarkers have been observed. Established muscle damage markers creatine kinase (CK) and myoglobin have been shown to elevate following a rugby game (Jones *et al.*, 2014; Smart *et al.*, 2008; Takarada 2003; West *et al.*, 2013), while inflammatory markers such as c-reactive protein (CRP) and interleukin-6 (IL-6) have all become significantly increased as well (Cunniffe *et al.*, 2010). Immunological (salivary immunoglobulin A (sIgA) and lymphocyte populations), hormonal (cortisol and testosterone) and haematological parameters have similarly been highlighted to change dramatically and occasionally provide a predictive game outcome (Banfi *et al.*, 2006; Cunniffe *et al.*, 2010; Elloumi *et al.*, 2003; Gaviglio *et al.*, 2014; Morgan 2011), while rugby union also induces changes in oxidative stress (Finaud *et al.*, 2006b), lactate and heart rate (Deutsch *et al.*, 1998).

Changes in both physiological and psychological elements not only result in a higher susceptibility to over-training or overreaching, fatigue and illness (Cunniffe *et al.*, 2011), but may also affect performance measures such as counter movement jumps, 1-repetition max and on-field performance. Over-training and reaching is considered an unwanted and inconvenient aspect of athletic training and performance that requires careful individual

monitoring of a player. It is a condition of chronic fatigue, underperformance, and an increased vulnerability to infection leading to recurrent infections (Budgett 1998). This phenomenon is one of the most feared complications for a player that can last weeks or months (Lehmann *et al.*, 1998; Urhausen and Kindermann 2002). Careful perception is advised when discriminating between this and over-reaching; a relatively short-term decrement in performance followed within a few days by a full recovery or improvement in performance (supercompensation)(Gleeson 2002). There is now a general consensus regarding the difference between functional overreaching (FO), non-functional overreaching (NFO) and overtraining syndrome (OTS) (Meeusen *et al.*, 2013). Its significance in rugby is based around under-recovery; a result of excessively prolonged or intense exercise, stressful competition, or other stresses which lead to progressive fatigue and underperformance (Budgett 1998). This may be a result of ignoring the signs of fatigue, heavy muscles and depression until performance is chronically affected (Dymment 1993). Attention to these factors is essential to today's modern era of professional rugby for player longevity and team performance.

### 1.3 IMMUNE SYSTEM

Immunity is split up into two distinct and interdependent components: the innate or “non-specific” immune system and the adaptive or “specific” immune system. Innate immunity is comprised of several components, 1) Inflammation, a non-specific response to tissue injury that becomes activated through chemical factors including cytokines, 2) the complement system, a group of inactive plasma proteins that when activated, bring about opsonisation, chemotaxis of phagocytic cells, cell lysis and agglutination, 3) innate leukocytes, including natural killer cells responsible for destroying virus infected cells, and lastly 4) interferon, a family of proteins that defends against viral infections (Sherwood 2008). Adaptive immunity however, mediated by T and B lymphocytes separated into antibody-mediated or cell-mediated, is a specialized system that efficiently eradicates a previously recognized foe.

Assessing the effect of exercise related stress on the immune system is imperative to alleviate the risk of recurrent infections and a decrease in performance. There is equivocal evidence surrounding the impact of illness on exercise performance. While some research identifies no change in performance between ill and healthy athletes (Pyne *et al.*, 2001; Weidner *et al.*,

1997), some have identified a decline in sport performance (Weidner 1994). With up to 90 % of all infections occurring in the mucosae in regard to microbial colonization and entry into the body (Brandtzaeg 2003), the majority of illnesses associated with diminished exercise performance are based around upper respiratory tract infections (URTI). Furthermore, the effect of illness on selected pulmonary, cardiac and skeletal muscle functions may lead to illness complications and protracted courses of URTI in athletes (Weidner 1994).

### 1.3.1 Exercise Effect

The relationship between exercise, immunity and exertion was first approached by Larrabee (1902). He recognized a large increase in leucocytosis of the inflammatory type in four marathon runners following a race. Since then, a vast array of exercise related studies have investigated the effect of various exercise related activities on selected markers of both the innate and adaptive immune systems. In athletes, respiratory viral infections represent the most prevalent and pathogenic form of infectious disease (Mathers *et al.*, 2008).

In 1994 (Nieman), the “J-curve” concept was developed (Fig. 1.1); a representation of infection risk based on exercise intensity. It describes exercise of a moderate intensity decreasing the risk of infection. This has been established by a number of studies identifying a 29 % risk reduction of URTI’s following two hours of moderate exercise compared to a sedentary lifestyle (Matthews *et al.*, 2002), a reduced risk of self-reported respiratory symptoms when completing moderate intensity exercise (Kostka *et al.*, 2000; Wong *et al.*, 2008), and a reduced number of days with a URTI in active people compared to sedentary controls (Nieman *et al.*, 2011). However competing in ultra-endurance (high-intensity) based events can cause a 100 – 500 % increase in the risk of infection in the weeks following (Nieman *et al.*, 1990; Peters *et al.*, 1996; Peters *et al.*, 1993; Peters *et al.*, 2010). This can equate to as much as 47 % of competitors experiencing a URTI (Robson-Ansley *et al.*, 2012), while intense exercise before or during an infection has been associated with greater morbidity and mortality (Ekblom *et al.*, 2006; Heath *et al.*, 1991).

Studies on animals have provided further robust information regarding the effect of exercise intensity on immune system function. Mice inoculated with a herpes simplex virus-1 or horses with influenza had severe deficiencies in immune defence in the high intensity group

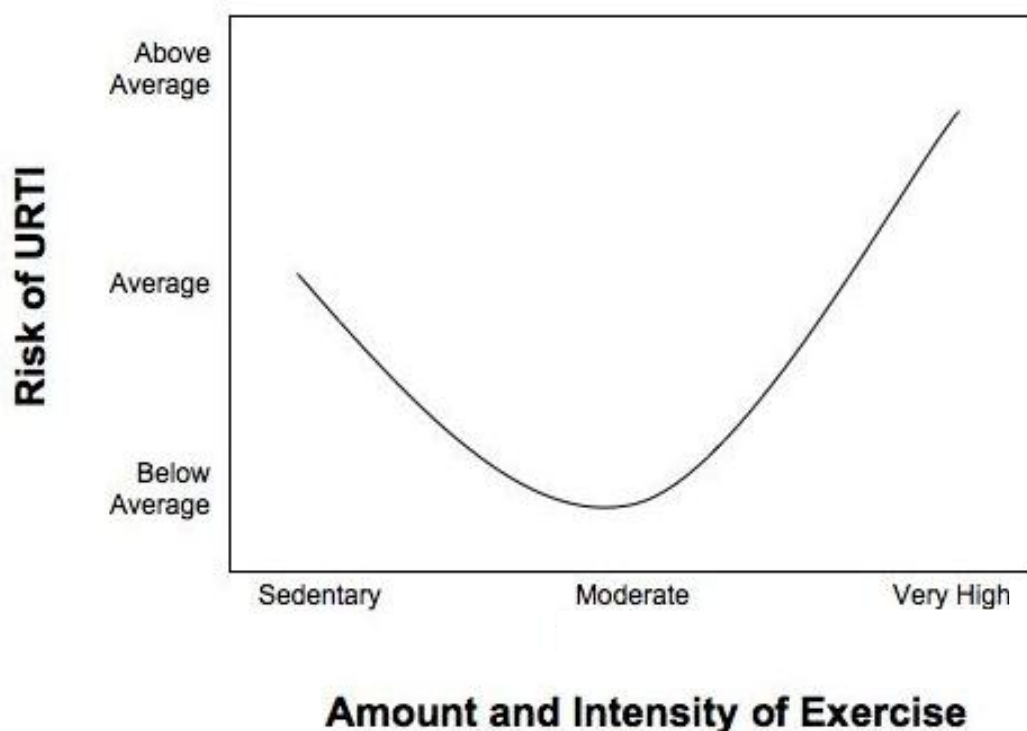
resulting in the higher probability of death or more severe symptoms, respectively (Davis *et al.*, 2004; Gross *et al.*, 1998). In contrast, a human study identified no relation between training volume and post-race incidence of URTI's (Ekblom *et al.*, 2006) which contradicts athletes completing > 97 km per week in training volume – doubling their odds of an infection compared to those completing < 32 km per week (Nieman *et al.*, 1990). This is corroborated by the higher level of URTI's observed in athletes completing > 7 hours per week of training (Gleeson *et al.*, 2013), basketball players during a camp (Moreira *et al.*, 2011) and athletes completing higher training loads (Gleeson *et al.*, 2012).

A similar concept has been developed following severe intensity exercise. The “open-window” concept (Fig. 1.2) was established postulating a link between immune changes, infection and exercise intensity (Hoffman-Goetz and Pedersen 1994; Pedersen and Ullum 1994). It describes a period in the time following intense exercise where the immune system is in a compromised state. This can result in a virus gaining entry that increases the risk of clinical and sub-clinical infection. The increased risk of infection and open-window concept observed following high intensity exercise can be attributed to changes in the components of the innate and adaptive immune systems. Resistance training has been shown to cause an increase in the apoptosis and migration of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes for up to 24 hours (Pereira *et al.*, 2012), alongside a decrease in their circulating concentration (Cardoso *et al.*, 2012). Post-exercise changes in immune parameters have also been identified following 90 minutes of intense cycling in “A” grade athletes. Total lymphocyte numbers decreased two hours post-exercise, natural killer (NK) cells decreased significantly for up to eight hours, and neutrophil phagocytic function decreased at six and eight hours post exercise (Kakanis *et al.*, 2010).

Immune parameters have also been identified as potential indicators of over-training or over-reaching. Several indices including neutrophil and monocyte oxidative burst, T-lymphocyte CD4<sup>+</sup>/CD8<sup>+</sup> ratio, mitogen-stimulated lymphocyte proliferation, antibody synthesis and NK cell cytolytic activity are sensitive to increases in the training load in well-trained athletes (Baj *et al.*, 1994; Gleeson *et al.*, 1995; Lancaster *et al.*, 2004; Robson *et al.*, 1999; Verde *et al.*, 1992). For example, six months of intense cycling training completing approximately 500 km per week resulted in significant decreases in absolute numbers of CD3<sup>+</sup> and CD4<sup>+</sup>

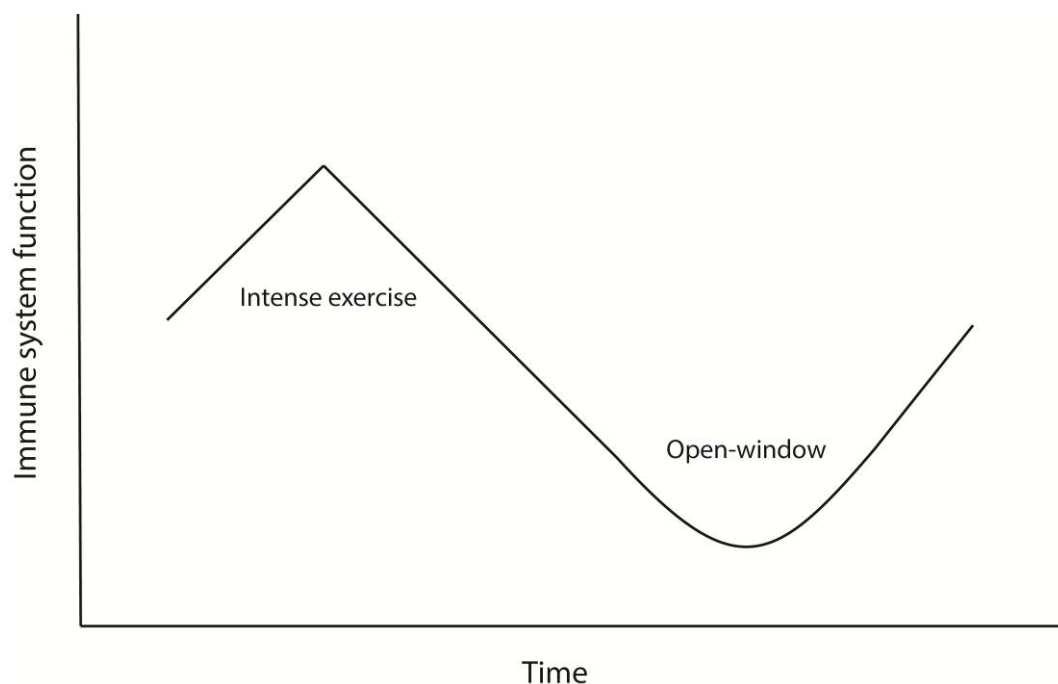
cells, diminished interleukin-2 (IL-2) production and reduced oxidative burst activity of neutrophils (Baj *et al.*, 1994).

The above changes highlight the effects of intense exercise. It is both duration and intensity dependent which can result in an increased susceptibility to URTI. The size and time course changes of separate immune parameters highlights the variability in their response to exercise. While some cells can become elevated, others decrease immediately over a period of up to 24 hours (Kakanis *et al.*, 2010). Work on rugby union has highlighted similar observations with total leukocytes and lymphocytes significantly increasing immediately post-game and remaining elevated for up to 14 hours. Additionally NK cell number decreased immediately post-game, while  $CD4^+$  and  $CD8^+$  remained unchanged and decreased respectively (Cunniffe *et al.*, 2010).





**Figure 1.1.** J-curve concept highlighting the risk of infection based on exercise intensity. Reproduced with permission as part of (Nieman 1994).



**Figure 1.2.** The “open-window” concept describes a period following intense exercise where host defence is compromised increasing the risk of infection. (Adapted from Pedersen et al. 1997).

### 1.3.2 Salivary Immunoglobulin A

Salivary immunoglobulin A is one of the most well studied markers of the mucosal immune system due to its relative ease of collection and extensively investigated response in relation to exercise. It can be used both as a marker of exercise intensity and indicator of both URTI's and over-training and reaching.

Salivary immunoglobulin A is a dimeric molecule used by the immune system as a “first line of defence” to identify and neutralise foreign objects through recognition of a unique part (antigen) of the foreign target (Bishop and Gleeson 2009; Litman *et al.*, 1993). The cell

surface receptor of immunoglobulins (Ig) prevents cell signalling and activation of an antigen, while the soluble effector molecules individually bind and neutralize antigens at a distance (Schroeder Jr and Cavacini 2010). Immunoglobulin A has also been shown to interact with the  $F_c$  receptor causing an antibody-dependent cell-mediated cytotoxicity degranulation of basophils and eosinophils and phagocytosis by macrophages and neutrophils (Snoeck *et al.*, 2006). It is produced largely in mucosal linings (Fagarasan and Honjo 2003) which constitutes 75 % of the total Ig's produced each day (Macpherson and Slack 2007).

There are two subclasses of IgA: IgA1 and IgA2. Immunoglobulin A1 is the predominant subclass in saliva (Gleeson and Pyne 2000) which is dependent on the nature of the antigen inducing its production (Simell *et al.*, 2006). As a result of Ig variability (Fanning *et al.*, 1996) which is controlled by somatic hypermutation of B-cells (Diaz and Casali 2002), the diversification of the antibody pool allows them to act on a variety of antigens that is controlled by a process known as affinity maturation (Neuberger *et al.*, 2000; Or-Guil *et al.*, 2007).

Salivary immunoglobulin A is produced by plasma cells (differentiated B lymphocytes) adjacent to ducts and acini of the salivary gland (Korsrud and Brandtzaeg 1980), whose transport (bound to its secretory component that provides protection against proteolytic degradation (Crottet and Corthésy 1998)) and function (first line of defence against microbial infection) across the epithelium can occur in three potential ways:

- prevention of pathogen adherence and penetration of the mucosal epithelium
- neutralization of viruses within the epithelial cells during transcytosis
- excretion of locally formed immune complexes across mucosal epithelial cells to the luminal surface (Lamm 1998).

Salivary immunoglobulin A secretion, like other salivary components (see saliva 1.9), is under the control of the autonomic nervous system (ANS). Innervated by both sympathetic and parasympathetic activation, alterations in their stimulation of salivary glands or the blood vessels that supply the glands are a primary mode of control (Bishop and Gleeson 2009). Therefore the increase observed during moderate intensity and high intensity exercise can be explained by a rat model that showed a 2.6 to 6 - fold increase in sIgA secretion (gland dependent) following stimulation (Carpenter *et al.*, 1998; Proctor *et al.*, 2000). B-cell

production of IgA and transepithelial transport are also limiting factors (Bosch *et al.*, 2002). The lack of change or reduction in sIgA secretion is more difficult to interpret. A rat model may explain the difficulties of this phenomenon when the decreased rate of sIgA secretion following 60 minutes of exhaustive exercise was coupled with a decrease in the IgA receptors (polymeric Ig receptor), alongside mRNA expression that is required for transepithelial transport (Kimura *et al.*, 2008). Moreover, a study examining the effect of quercetin on a 160 km endurance race identified a significant decrease in sIgA secretion rate but not concentration (Henson *et al.*, 2008). This is explained by the flow rate being related to the removal of parasympathetic vasodilatory influences. Under reflex conditions, vasoconstriction is not responsible for altered saliva volume due to the lack of vasoactive nerve fibre stimulation (Bishop *et al.*, 2006; Bosch *et al.*, 2002; Proctor and Carpenter 2007).

Other influences have also been investigated, with the cold dry-air in a cross country ski race being attributed to the decreased secretion rate of sIgA (Tomasi *et al.*, 1982). However there is equivocal evidence surrounding this theory with some reporting differences in sIgA between groups exercising at different temperatures (Walsh *et al.*, 2002), while some show no change (Housh *et al.*, 1991). Changes in cortisol have also been suggested as a possible influence on immunosuppression when basketball players analysed throughout a season showed an inverse correlation between salivary cortisol and sIgA secretion rates (He *et al.*, 2010). A relationship has also been noticed in young adults (Hucklebridge *et al.*, 1998), although some studies have identified changes in sIgA concentration and secretion rate without a change in plasma or salivary cortisol (Allgrove *et al.*, 2008) making this theory unlikely.

### 1.3.3 sIgA Response to Exercise

A selective deficiency in sIgA has been noted in those with a high incidence of infection (Hanson *et al.*, 1983) or poor saliva flow rate (Fox 1985). This is in contrast to Ammann and Hong (1971) who stated IgA deficiency is relatively common and not usually associated with a markedly impaired resistance to infection. Even though several diseases are associated with an increased sIgA secretion rate (Elkon *et al.*, 1983; Kalsi *et al.*, 1983; Procaccia *et al.*, 1987) and low incidence of URTI's (Rossen *et al.*, 1970), the common immunosuppression observed in athletes does not necessarily mean they are immunocompromised in their

response to common antigens (Mackinnon *et al.*, 1987). Equivocal evidence surrounds this topic with suggestions the antiviral defence mechanisms of athletes may be compromised which leads to a decrease in performance (Gleeson *et al.*, 2002; Levando *et al.*, 1988).

The research on the effect of exercise on sIgA is expansive and extensively reviewed (Bishop and Gleeson 2009). Salivary immunoglobulin A is unquestionably exercise intensity dependent, with moderate intensity protocols failing to elicit any significant post-exercise changes (Bratthall and Widerstrom 1985; McDowell *et al.*, 1991; Tharp 1991; Walsh 1999). For example, 29 active males ran on a treadmill to exhaustion that resulted in the mean sIgA significantly decreased post-exercise. However five of the participants showed a 1.3 – 53.3 % increase (McDowell *et al.*, 1992). This highlights a further effect of inter-individual variation that is physiologically dependent. Similar studies have also identified no change in sIgA following an elite soccer match (Thorpe and Sunderland 2012), intensive tennis training (Gomes *et al.*, 2013), a collegiate rugby game (Koch *et al.*, 2007), jiu-jitsu matches (Moreira *et al.*, 2012) or resistance exercise (Carlson *et al.*, 2013; Roschel *et al.*, 2011). The subjects involved in these studies did not fall into an immunocompromised state that may be related to several physiological and non-physiological factors.

Meanwhile, there are several studies that have identified immediate post-exercise decreases in sIgA. Ultra-endurance events are considered one of the most physiologically demanding in the world that results in an immediate post-exercise suppression (Gill *et al.*, 2014; Henson *et al.*, 2008; Tauler *et al.*, 2013). Similarly, cycling for two hours at 70%  $\dot{V}O_{2max}$  (Walsh *et al.*, 2002), cross country skiing for 50 km (Tomasi *et al.*, 1982), cycling supra-maximally on an erg (exercycle) for 5 x 60 s with five minutes rest between efforts (MacKinnon and Jenkins 1993), and competing in a triathlon (Steerenberg *et al.*, 1997) all cause the athlete or subject to become immunocompromised.

Salivary immunoglobulin A monitoring may also be useful in determining the risk of infection (Pedersen and Nieman 1998; Pyne and Gleeson 1998) and excessive training in athletes (Mackinnon and Hooper 1994; Shephard and Shek 1998). Decreased levels of sIgA have been associated with stale, underperforming and over-trained athletes (Budgett 1998; Foster 1998; Gleeson *et al.*, 1999). In a swimming study, significant decreases in sIgA during six months of training were observed (Kormanovski *et al.*, 2010) which is similar to that previously noted (Gleeson *et al.*, 2000; Gleeson *et al.*, 1999). Elite kayakers have also

showed a 27 – 38 % decrease in sIgA secretion rate after each session of training over three weeks (Traeger Mackinnon *et al.*, 1993). In contrast, no change in sIgA secretion rate was observed in 20 female division one soccer players during a 13 week season (Vardiman *et al.*, 2011), which is similar to the lack of change in pre-race sIgA in competitors completing 10 marathons in 10 days (Southworth *et al.*, 2013). While sIgA may provide some insight into potential over-training and reaching and risk of infection, it seems to be biased regarding the type of exercise and the individuals involved.

There is also a general consensus in the literature that decreased levels of sIgA are associated with an increased incidence of URTI's (Walsh *et al.*, 2011). As the most common form of infection present in athletes, URTI's present an unwanted and challenging aspect in addition to the normal rigors of training. An increase in URTI's has been linked to decreased sIgA concentrations following a soccer match (Mortatti *et al.*, 2012), basketball game (Moreira *et al.*, 2011), at least three hours a week endurance training (Gleeson *et al.*, 2012), American Football (Fahlman and Engels 2005), swimming (Gleeson *et al.*, 1995) and rugby union (Cunniffe *et al.*, 2011; Yamauchi *et al.*, 2011). In contrast, no relationship between URTI incidence and sIgA following a 84 km ultra-marathon was observed (Peters *et al.*, 2010), while similar observations have been seen in soccer (Vardiman *et al.*, 2011), military training (Tiollier *et al.*, 2005) and yachting (Neville *et al.*, 2008). In the latter case, it was noted that if a subject who was not suffering from or recovering from a URTI had a sIgA value less than 40 % of their mean healthy sIgA level, that they had a 50 % chance of contracting a URTI within three weeks. Differences may be attributable to the self-reporting of symptoms vs. physician diagnosis, the reporting of sIgA as a concentration or secretion rate, collection protocols, or the intensity difference which is difficult to differentiate between types of exercise.

It is evidently clear sIgA is a reliable indicator of immune system function in athletes whose changes can represent potential susceptibility to clinical or sub-clinical infection based on the intensity and individual physiology of the subject. In terms of rugby related stress which is considered one of the most physically demanding games, acute and longitudinal changes in sIgA may be effective at identifying those players at risk.

## 1.4 INFLAMMATION

The inflammatory response is a non-specific integral part of the innate immune system responsible for clearing the inflamed area of infectious and toxic agents and tissue debris by phagocytic and non-phagocytic means (Sherwood 2008). During exercise and particularly impact related sport, tissue damage is a common theme that results in the activation of the inflammatory system.

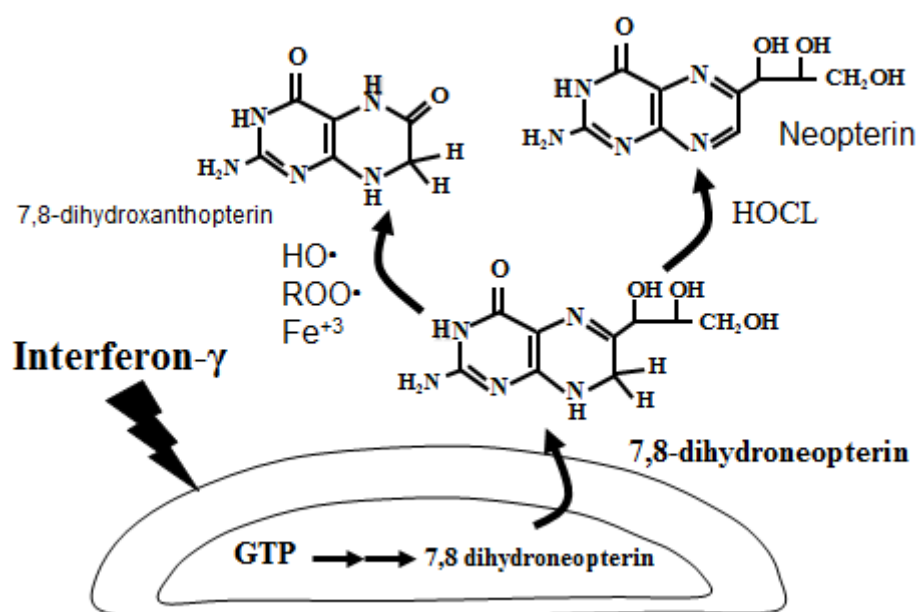
### 1.4.1 Exercise-Induced Inflammatory Effect

Exercise of various intensities can cause a transient increase in markers of inflammation. Interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and CRP are exceptionally common markers that become elevated following exercise. Interleukin-6 has been shown to increase from 0.76 pg/mL to 10.89 pg/mL following a 24 hour simulated laboratory marathon as well as a six day adventure race (Wallberg *et al.*, 2011). This is comparatively mild to the 8000-fold increase in IL-6 following a 246 km ultra-endurance marathon (Margeli *et al.*, 2005). Additionally, IL-6 mRNA expression is up-regulated during exercise (Nieman *et al.*, 2003b) which is intensity and duration dependent (Pedersen *et al.*, 2003). However its specificity to exercise induced tissue damage is questionable as a result of its rise irrespective of muscle damage (Febbraio and Pedersen 2002) and its newly defined function as a “myokine” (Febbraio and Pedersen 2005). Similarly, IL-1 $\beta$  and TNF- $\alpha$  become elevated post-marathon (Moldoveanu *et al.*, 2000; Ostrowski *et al.*, 1999) and after short intense exercise (Espersen *et al.*, 1990). C-reactive protein, the first acute phase protein to be described that is a sensitive marker of inflammation (Pepys and Baltz 1983), has been significantly increased following ultra-endurance exercise (Scherr *et al.*, 2011; Tauler *et al.*, 2013) rugby union (Cunniffe *et al.*, 2010) and soccer (Ispirlidis *et al.*, 2008).

### 1.4.2 Neopterin and 7,8-dihydroneopterin

Interferon- $\gamma$  (IFN- $\gamma$ ), produced from NK and NK T cells and CD4<sup>+</sup> and CD8<sup>+</sup> cells once antigen-specific immunity develops (Schoenborn and Wilson 2007), stimulates macrophage production of 7,8-dihydroneopterin from guanosine tri-phosphate (GTP) via up-regulation of GTP-cyclohydrolase 1 (GTPCH-1) (Schoedon *et al.*, 1986). 7,8-dihydroneopterin triphosphate; the enzymatic breakdown product of GTP (Schoedon *et al.*, 1986) is an

intermediate in the production of tetrahydrobiopterin ( $\text{BH}_4$ ) that is cleaved by intracellular phosphatases (Schoedon *et al.*, 2005) in macrophages specifically (Wirleitner *et al.*, 2002). 7,8-dihydroneopterin is secreted from the activated macrophage into the intracellular spaces and finally the plasma (Giesege *et al.*, 2008a), where proton abstraction from carbon-7 and nitrogen-8 signifies oxidation to the highly fluorescent neopterin (NP) by hypohalous acids such as hypochlorous acid (HOCL) (Fig. 1.3) (Giesege *et al.*, 2000; Schraufst tter *et al.*, 1990; Widner *et al.*, 2000).



**Figure 1.3.** Neopterin and 7,8-dihydroneopterin production.

Hypochlorite is generated by neutrophils and to a lesser extent macrophages during inflammation (Halliwell and Gutteridge 1999; Pullar *et al.*, 2000) suggesting much of the NP

measured in plasma has come from these sites. To date it is the only known compound capable of oxidizing 7,8-dihydroneopterin to NP *in vivo*, although it is widely accepted other potential mechanisms exist that are yet to be described. One such mechanism that may be related to impact-induced muscle damage is the oxidizing potential of the heme containing protein myoglobin. With significant increases observed following rugby union (Takarada 2003) and its proven ability to cause lipid peroxidation (Giulivi and Cadenas 1998), myoglobin oxidation may be an alternative mechanism responsible for a hypothesized increase in NP following rugby union.

Regardless, the central role of IFN- $\gamma$  communication between T-cells and macrophages with the subsequent release of 7,8-dihydroneopterin and its oxidized form NP, make them ideal measurements for gauging immune activation (Wachter *et al.*, 1989). An experiment found that direct injection of IFN- $\gamma$  results in a significant and sustained rise in plasma NP (Müller *et al.*, 1991) which provides further conclusive evidence of the mechanism.

Neopterin has been implicated in several pathological diseases and conditions including HIV, cancer, arthritis, infections, septicaemia, multiple sclerosis, atherosclerosis, allograft recipients and tuberculosis (Aulitzky *et al.*, 1988; Baydar *et al.*, 2009; Beckham *et al.*, 1992; Eisenhut *et al.*, 2011; Giesege *et al.*, 2008a; Hoffmann *et al.*, 2003). To a lesser extent, it has also been reported to possess pro-oxidant properties (Hoffmann *et al.*, 2003), where its balance with 7,8-dihydroneopterin in atherosclerotic plaques can influence its progression (Giesege *et al.*, 2008a; Giesege *et al.*, 2008b), while also being a significant tool for blood and organ donor screening (Fuchs *et al.*, 1983). Its serum concentration meanwhile, is dependent on age and gender while being modified by race, body mass index (BMI) and body fat percentage (Spencer *et al.*, 2010).

7,8-dihydroneopterin's properties depend on the concentration, chemical environment and oxidants it can react with. It has been reported to have anti-oxidant and pro-oxidant properties (Giesege *et al.*, 1995). 7,8-dihydroneopterin inhibits oxidized low-density lipoprotein (oxLDL) induced cell death in U937, THP-1 and human macrophages (Baird *et al.*, 2005; Giesege and Cato 2003). It has been demonstrated to be a potent scavenger of superoxide, peroxy and nitrogen centred radicals (Baird *et al.*, 2005; Giesege and Cato 2003; Oettl *et al.*, 2004; Weiss *et al.*, 1993) and shown to induce apoptosis (at high mM



concentrations only) in several cell lines due to increased oxidative stress (Wirleitner *et al.*, 2003).

#### 1.4.2.1 Detection Methodology

The quantification of NP and 7,8-dihydroneopterin can provide a total measurement of macrophage activation and the level of associated inflammation. As a diagnostic tool in several diseases and markers of exercise-induced immune activation, the measurement requires a rapid and cost-effective methodology. The detection of urinary NP was first described in the late 1970's (Wachter *et al.*, 1979) which was closely followed by Fukushima and Nixon (1980) who both used a C<sub>18</sub> reverse phase method employing a phosphate buffer and methanol/H<sub>2</sub>O mobile phase, respectively. They also measured the dihydro- forms following acidic and basic iodine oxidation because of their lack of fluorescence.

Whilst several methods are available for NP and 7,8-dihydroneopterin quantification including enzyme linked immunosorbent assay (ELISA), dipstick and radioimmunoassay (RIA) (Bühner-Sekula *et al.*, 2000; Werner *et al.*, 1987), their sensitivity and processing speed does not account for their relatively expensive nature. High performance liquid chromatography (HPLC) is the preferred method of detection due to its inexpensive sampling costs after initial set-up and auto-sampling ability. Urinary NP and 7,8-dihydroneopterin detection are most commonly assayed using the reverse phase method developed by Hausen *et al.* (1982) which was later updated in 1992 (Fuchs *et al.*, ). It uses a phosphate buffer between pH 6 to 7, where 100 µL of urine is diluted with 1000 mL of a 0.015 mol/L phosphate buffer (pH 6.4) containing 2 g/L ethylenediaminetetraacetic acid (EDTA). Chromatography is isocratically performed using the dilution buffer without the EDTA with monitoring of NP done by its native fluorescence at 353 nm excitation and 438 nm emission wavelengths with a flow rate of 1 mL/min. Pre-column sample preparation is kept to a minimum because of a lack of protein present. In comparison, plasma, serum and cerebrospinal fluid requires careful precipitation of proteins by ethanol (Krcmova *et al.*, 2011) or acetonitrile (ACN) (Flavall *et al.*, 2008). de Castro *et al.* (2004) proposed an alternative method that used two coupled reverse phase columns, 150 mM sodium phosphate buffer pH 4 at a flow rate of 0.8 mL/min using various UV wavelengths from 353 – 390 nm. While they claimed it was a reliable and efficient alternative, Schroecksnadel *et al.* (2006)

appealed the results stating the large variation in comparison to previously published work was due to the inability to distinguish NP from 7,8-dihydroneopterin.

#### 1.4.2.2 Exercise Effect

Neopterin has been routinely measured in plasma and urine as a marker of immune activation following various forms of exercise including running (Rokos *et al.*, 1987), rowing (Jakeman *et al.*, 1995), cycling (Deetjen *et al.*, 1997) and triathlon (Margaritis *et al.*, 1997). Deetjan *et al.* (1997) monitored subjects in an eight hour alpine cycling race that resulted in significant and sustained (48 hours) increases in NP immediately post-race. Similar kinetics were observed following a two and a half hour run (Dufaux and Order 1989), a 20 kilometre run completed in under two hours (Sprenger *et al.*, 1992), five hours intense running (Tilz *et al.*, 1993) and 67 km ultra-marathon (Schobersberger *et al.*, 2000). Schobersberger (2006) also observed a rise in serum NP, CK and CRP following a downhill marathon run, whilst NP also has the potential to identify over-training or reaching. Jakeman *et al.* (1995) observed one third of an increase in the NP/creatinine ratio compared to pre-training levels in 27 elite rowers whose training had increased in the four weeks leading up to the Olympics.

The measurement of NP in exercise has declined over the past decade. In an investigation into NP excretion in individuals who participated in the Race across America in 2007, NP was shown to increase steadily, peaking at day four. It then began to drop toward the end of the race where at day seven there were no differences between the competitor and support person. There was also large inter-individual differences and a correlation with power output indicating NP excretion is intensity dependent (Moser *et al.*, 2008). Furthermore, NP in conjunction with muscle damage and oxidative stress markers were observed to significantly increase following a 90 kilometre marathon race in Brazil (Dantas de Lucas *et al.*, 2014). In contrast, an investigation of 15 trained runners following a marathon observed no significant increase up to 34 hours afterwards (Gunga *et al.*, 2002). Whilst this may be related to the fitness level of the individuals involved, no difference in the NP change was observed between trained and un-trained subjects following one hour of cycling at 60 %  $\dot{V}O_2\text{max}$  (Smith *et al.*, 1992). This may of course be slightly altered because of the moderate intensity associated with that level of maximal  $O_2$  uptake.

Furthermore, there are several issues that can be drawn from the current literature regarding NP quantification measurement as an indicator of inflammation from exercise. Traditionally papers have investigated NP alone and neglected to measure 7,8-dihydroneopterin (Dantas de Lucas *et al.*, 2014; Dufaux and Order 1989; Sprenger *et al.*, 1992). To gain a true understanding of macrophage activation, total NP (NP + 7,8-dihydroneopterin) has to be measured. Therefore, the papers who have recognized a NP increase may in fact only be measuring a change in oxidative stress. With NP concentrations approximately a third of total NP (Fuchs *et al.*, 1989b), it is highly possible the increased NP observed following exercise is a result of an increased oxidation of 7,8-dihydroneopterin and not a result of increased macrophage activation. The mentioned studies did however identify a simultaneous rise in TNF- $\alpha$  which subsequently suggests an inflammatory reaction has ensued as a result of the exercise. Meanwhile, NP and 7,8-dihydroneopterin have not been measured in a sport that is focused around high force impacts and collisions that are known to cause muscle damage and induce changes in inflammatory and endocrine markers (McLellan *et al.*, 2010; Smart *et al.*, 2008).

## 1.5 MUSCLE DAMAGE

Exercise-induced muscle damage from contact related sport can occur in two ways. Firstly the eccentric loading of the muscle causes sarcomere disruption in myofibrils and damage to the excitation-coupling system (Proske and Morgan 2001). Secondly blunt force trauma causes structural integrity loss of the muscle cell membrane which results in the release of intramuscular constituents into the plasma. Muscle damage is followed by regeneration, repair and adaptation, and is associated with primary inflammation, cell proliferation, apoptosis and the formation of scar tissue (Huard *et al.*, 2002). This subsequently reduces muscle force production (Connolly *et al.*, 2003; Howell *et al.*, 1993) and motor neuron activation and sensitivity (Prasartwuth *et al.*, 2005). Significant concern arises when blunt force trauma occurs from a high force impact common in contact sport that can result in catastrophic loss of muscle cell integrity resulting in significant health concerns including rhabdomyolysis (Brown 2004).

### 1.5.1 Exercise-Induced Muscle Damage

Quantification of skeletal muscle damage severity following exercise is one of the more common measurements of stress. Markers generally include myoglobin, CK, carbonic anhydrase III (CAIII), skeletal troponin I (sTnI), lactate dehydrogenase (LDH), aspartate aminotransferase and aldolase. Careful consideration following intense exercise has to be given to the effect of oxidative stress on muscle damage which will be discussed in a later section. For the purpose of this section, only direct intramuscular constituents will be discussed.

Serum concentrations of CK have been significantly increased following running of varying durations and intensities (Kanter *et al.*, 1988; Ostrowski *et al.*, 2000; Rahimi *et al.*, 2010a), eccentric loading (Nosaka and Clarkson 1996; Nosaka and Newton 2002) and used as a marker of overload in professional soccer players (Lazarim *et al.*, 2009). They all seem to indicate its release is intensity and duration dependent (Kanter *et al.*, 1988; Strachan *et al.*, 1984). Specifically, its concentrations tend to become more significantly elevated with physical impact (Ehlers *et al.*, 2002; Hoffman *et al.*, 2002) while identifying rugby and rugby league as sports that induce severe muscle damage (Cunniffe *et al.*, 2010; McLellan *et al.*, 2010, 2011a; Smart *et al.*, 2008). Meanwhile, LDH, CAIII and sTnI have all been shown to increase following a marathon or eccentric loading exercise (Fu *et al.*, 2009; Kobayashi *et al.*, 2005; Nosaka *et al.*, 1992; Sorichter *et al.*, 1997). Whilst these markers provide an effective means of understanding the level of muscle damage associated with exercise, they are predominantly quantified in serum or plasma and have comparatively slow kinetics which makes them obsolete for the acute and immediate nature of this research.

### 1.5.2 Myoglobin

Myoglobin is a 17 kDa single-chain oxygen-carrying hemoprotein restricted to cardiomyocytes and oxidative skeletal myofibers. The detection of myoglobin in the bloodstream is a diagnostically relevant measurement indicative of muscle damage (Nelson and Cox 2000). It facilitates O<sub>2</sub> delivery from the intra-capillary erythrocyte to the mitochondria in order to maintain oxidative phosphorylation for myocardial contractility. Myoglobin deficient mice have been found to be viable with preserved cardiac function due to their ability to mount a complex compensatory response involving increased vascularization, induction of the hypoxia gene program, reduced cell width, elevated

hematocrit and increased coronary flow and coronary flow reserve (Grange *et al.*, 2001; Mammen *et al.*, 2003; Meeson *et al.*, 2001). Myoglobin's other potential roles also include acting as an O<sub>2</sub> reservoir, a cytoprotective protein against reactive oxygen species (ROS), and as a modulator of nitric oxide (NO) (Flögel *et al.*, 2001; Kojda and Kottenberg 1999; Trochu *et al.*, 2000).

Myoglobin's use in the clinical field is critical for diagnosis of patients with rhabdomyolysis (Feinfeld *et al.*, 1992) or acute kidney injury (Zager and Burkhart 1997). It's extremely fast elimination kinetics (Suzuki *et al.*, 1999) and relatively fast time to peak in comparison to CK (Mikkelsen and Toft 2005) provides a sharper response and immediate opportunity to gauge muscle damage severity. It is a sensitive and credible muscle damage marker of choice in rugby union due to the number of high force impacts (Smart *et al.*, 2008). It is rapidly filtered by the glomeruli and reabsorbed by the proximal tubules where it is catabolized as a result of its small globular size (Bagley *et al.*, 2007; Hamilton *et al.*, 1989). When the filtered load exceeds the re-absorptive capacity of the tubule, myoglobin spills over into the urine, colouring it red (Beetham 2000; Don *et al.*, 1997). However it is only when concentrations exceed 100 mg/dL that urine becomes discoloured by myoglobin (Gabow *et al.*, 1982). This is corroborated by a study on naval officers who demonstrated concentrations from 3.2 – 410 mg/L following training exercises where coffee-brown discolouration was noted (Smith 1968).

Furthermore, urinary myoglobin is notorious for its instability. Temperature, pH, unidentified urinary compounds smaller than 10kDa, short half-life, metabolism to bilirubin, dissociation at acidic pH and time to analysis have all been identified as causes for myoglobin instability (Alterman *et al.*, 2007; Chen-Levy *et al.*, 2005; Gabow *et al.*, 1982; King *et al.*, 2010; Naka *et al.*, 2005; Wu *et al.*, 1994). It has been recommended that urine samples be adjusted to an alkaline pH (8 - 9.5), analysed immediately with avoidance of multiple freeze-thaw cycles to eliminate potential loss (Chen-Levy *et al.*, 2005).

### 1.5.2.3 Exercise Effect

Similar to other muscle damage markers, myoglobin release is intensity and duration dependent. A study investigating the effects of 25 triathletes in competition, noticed those

with the fastest finishing time had the highest myoglobin concentration (Thomas and Motley 1984). In comparison, during a 48 hour adventure race, myoglobin was observed to continually increase throughout the duration (Wichardt *et al.*, 2011). Myoglobin has also been shown to increase in response to intense resistance exercise resulting in black discolouration of the urine and eight days in hospital (Moeckel-Cole and Clarkson 2009), downhill treadmill and outdoor running (Peake *et al.*, 2005; Sorichter *et al.*, 1999), as well as duathlon, ironman and cycling (Neubauer *et al.*, 2008; Sugama *et al.*, 2012; Suzuki *et al.*, 1999). Relevant to this research, Takarada (2003) observed a significant increase in plasma myoglobin concentration following two games of elite amateur rugby union that correlated with the number of impacts each player experienced. This implies that the blunt force trauma of the impacts, whether they are tackles, ball carries or ruck related, seem to cause ultra-structural damage to the muscle cells resulting in myolysis rather than damage through eccentric loading.

The elimination kinetics of myoglobin are unique characteristics that provide an advantage over other markers. Cycling for 90 minutes at 90 watts (W) saw myoglobin concentrations peak at both one and three hours post (Suzuki *et al.*, 1999). Running on a treadmill at a gradient of - 10 % also resulted in myoglobin peaking between one and three hours (Peake *et al.*, 2005), while myoglobin also peaked 45 minutes following the cessation of rugby play (Takarada 2003). In contrast, sustained myoglobin increases have been observed up to 24 hours after an ironman race that also showed signs of increase in certain individuals as much as 19 days later; a potential sign of impaired recovery (Neubauer *et al.*, 2008). This elevation has been similarly observed 48 - 72 hours following six sets of 85 % maximum voluntary contractions (Tseng *et al.*, 2012). A cooling aid was applied in the latter study that may have delayed recovery. Based on the literature, myoglobin may provide an instantaneous assessment of muscle damage severity that is superior to the delayed rise in other conventional markers (Cunniffe *et al.*, 2010).

Exercise induced myoglobin release can also contribute to the syndrome known as exertional rhabdomyolysis. This is a potentially life-threatening syndrome characterised by the breakdown and necrosis of striated skeletal muscle resulting in the subsequent release of potentially toxic intracellular contents into the systemic circulation (Giannoglou *et al.*, 2007; Khan 2009; Sauret *et al.*, 2002). This can occur directly through blunt force trauma that

directly disrupts the cell membrane resulting in release of its constituents, or through an increase in intracellular free ionized calcium to a level much higher than normal in the cytoplasm and mitochondria (Giannoglou *et al.*, 2007). It has been observed in several sports including American Football, swimming, body-building and running (Clarkson 2007; Do *et al.*, 2007; Galvez *et al.*, 2008; Kraemer *et al.*, 2013), with myoglobin often contributing to the detrimental effects. Myoglobinuria however does not occur without rhabdomyolysis, but rhabdomyolysis does not necessarily lead to visible myoglobinuria (Khan 2009).

Acute renal failure (ARF) is a complication of rhabdomyolysis that is dependent on the severity and duration of renal dysfunction. It can lead to chronic renal failure, damage to the heart or nervous system, and death (Bagley *et al.*, 2007; Hamilton *et al.*, 1989; Huerta-Alardín *et al.*, 2005). Myoglobin has been associated with renal dysfunction through several possible mechanisms. If levels exceed the protein-binding capacity of the plasma, myoglobin can precipitate in the glomerular filtrate. In conjunction with uric acid, this can lead to intraluminal casts, increased intra-tubular pressure, and subsequently decreased glomerular filtration rate (Don *et al.*, 1997; Huerta-Alardín *et al.*, 2005; Vanholder *et al.*, 2000; Zutt *et al.*, 2014). It also has nephrotoxic effects at acidic pH where it dissociates into ferrihaemate and globin which potentiates acute tubular necrosis (Khan 2009; Naka *et al.*, 2005), alongside the heme group enhancing renal vasoconstriction and ischemia through activation of the cytokine cascade (Beetham 2000; Huerta-Alardín *et al.*, 2005). Together with the large increases in myoglobin reported in contact related sports, these mechanisms may contribute to ARF in professional athletes.

## 1.6 PSYCHOPHYSIOLOGICAL STRESS

Maintaining a homeostatic equilibrium is essential for survival. Defined as conditions where an environmental demand exceeds the natural regulatory capacity of an organism (Koolhaas *et al.*, 2011), the effect of an internal or external stimuli is regulated through a series of biochemical processes used to maintain a “steady-state”.

Exercise-induced stress can be separated into two distinct mechanisms; physiological and psychological. Stimuli can include the mechanical action of exercise, where in contact related sport such as rugby union, major physical trauma from impacts or the abundance of

eccentric and concentric muscle contractions can contribute to a shift in the homeostatic balance. Similarly, the stress associated with performing or competing against an opponent can equally cause a dramatic change and the activation of several key processes. This is described succinctly by Mazur (1985): “individuals who are the most resistant to the “stress” of competition increase their chance of winning, achieving dominance, or maintaining a desirable status or rank”. It is therefore imperative for professional athletes to control stress to gain maximum advantage in the field of competition.

Several key biological markers have been investigated as potential indicators of exercise-induced stress that were not included in this study. Salivary alpha-amylase is responsible for the hydrolysis of polysaccharides (Papacosta and Nassis 2011), which is proposed as a non-invasive marker of SAM axis activation during the stress response to physical exertion (Bishop and Gleeson 2009; De Oliveira *et al.*, 2010; Strahler *et al.*, 2010a). Adrenaline and nor-adrenaline significantly increase following intensity and duration dependent exercise (McMurray *et al.*, 1987; Sagnol *et al.*, 1990; Schwarz and Kindermann 1989), whilst corticotropin-releasing hormone (CRH) and adrenocorticotrophic hormone (ACTH) have been shown to increase either in direct response to or anticipation of exercise (Elias *et al.*, 1991; Raastad *et al.*, 2000).

### 1.6.1 Cortisol

Cortisol is a steroid hormone or glucocorticoid synthesised in the zona fasciculata layer of the adrenal gland whose secretion is controlled by the direct stimulation of the HPA. Two key axes are responsible for stress management; the HPA and the SAM. Both represent a complex signalling strategy between the central nervous system and endocrine system to regulate the homeostatic equilibrium in response to stress. The HPA is a series of direct influences and feedback interactions between three glands: the hypothalamus, pituitary and adrenal glands. In anticipation of or during exercise, stimulation of the hypothalamus causes an increase in amplitude of CRH and arginine-vasopressin (AVP) secretion (Tsigos and Chrousos 1994) from the anterior hypothalamus that directly triggers the release of ACTH from the anterior lobe of the pituitary gland. Carried in the blood, ACTH then triggers the synthesis of cholesterol derived cortisol from the adrenal gland (Horrocks *et al.*, 1990). Controlled by the 11-beta hydroxysteroid dehydrogenase system (Tomlinson *et al.*, 2004),



cortisol is then dispersed to all H<sub>2</sub>O systems of the body to regulate certain biological systems in response to the exercise stress.

Cortisol has several biological properties that it exerts initially through ligand binding of its ubiquitous cytoplasmic receptors (Smith and Toft 1993). These translocate into the nucleus following ligand binding where they interact with specific glucocorticoid responsive elements (GREs) within DNA to activate appropriate hormone-responsive genes (Pratt 1989). Alongside its stimulation of gluconeogenesis and glycogen synthesis in the liver and its ability to inhibit protein synthesis and stimulate protein degradation in peripheral tissues (Garrett and Grisham 2005), it also has immunosuppressive effects. One of the major concerns of cortisol in professional and recreational athletes is this immunosuppressive, catabolic and protein synthesis inhibitory effect. Cortisol has been shown to suppress both CD4 and CD8 T-cells (Shinkai *et al.*, 1996) as well as regulating degranulation (Peake 2002), ROS production (Tomchek *et al.*, 1991) and mobilization of neutrophils (Suzuki *et al.*, 2000). Furthermore, cortisol also has the ability to inhibit DNA replication and mitosis and repress the formation of antibodies and lymphocytes (Garrett and Grisham 2005; Posey *et al.*, 1978). This was evident following three hours of intense exercise where a strong correlation ( $r = 0.63$ ) was observed between post-exercise cortisol concentrations and lymphocyte apoptosis (Krüger *et al.*, 2011).

Cortisol secretion can be stimulated in response to several biological variables that alter its normal diurnal rhythm. This is a potential limitation when assessing the effects of exercise, which can be controlled by careful sample collection protocols. Under resting conditions, the diurnal rhythm is controlled by the amplitude of the CRH and AVP pulses. These are greatest in the early morning hours (Horrocks *et al.*, 1990), or through stimulation by other specific cytokines and hormones that are stress intensity dependent (Hinson 1990; Ottenweller and Meier 1982). These other influences can include caffeine which directly acts to stimulate adrenaline and nor-adrenaline secretion (commonly seen in exercise related studies (Lovallo *et al.*, 2006)), sleep deprivation (Leproult *et al.*, 1997), burnout (Wingenfeld *et al.*, 2009), severe trauma (Offner *et al.*, 2002) and anorexia nervosa (Haas *et al.*, 2009). It is also commonly observed in certain psychiatric disorders (Young *et al.*, 2004) and “stressful” situations. A study investigating the effects of a potential life-threatening event noticed dramatic cortisol elevations in men preparing for their first skydive (Chatterton *et al.*,

1997) as well as men participating in public speaking and mental arithmetic in front of an audience (Kirschbaum *et al.*, 1995). Cushing's Syndrome, characterized as hyper-cortisolism through abnormal secretion of ACTH from an overly developed pituitary gland, is diagnosed through cortisol quantification (Papanicolaou *et al.*, 2002).

#### 1.6.1.1 Exercise-Induced Changes

Salivary cortisol has been used in exercise related studies due to its ease of collection and representation of the free component in blood. Most serum cortisol is bound to proteins including cortisosteroid binding protein and albumin, whereas free cortisol passes relatively obstruction free through plasma membranes (Boron and Boulpaep 2009) allowing for rapid availability and ease of measurement. It has been used to assess the acute effect of a sport or exercise (Hough *et al.*, 2011; McLellan *et al.*, 2010), identify athletes in a state of over-training (Filaire *et al.*, 2013; O'Connor *et al.*, 1989; Tsai *et al.*, 2012), the recovery of an athlete (Elloumi *et al.*, 2003), or used simultaneously with testosterone to predict performance (Cormack *et al.*, 2008; Filaire *et al.*, 2001; Passelergue and Lac 2012).

Professional rugby league has been observed to cause significant increases in salivary cortisol (McLellan *et al.*, 2011b), which is similar to the post-exercise increases following soccer (Thorpe and Sunderland 2012), ultra-endurance marathon (Gill *et al.*, 2014), synchronized swimming (Tan and Long 2010), golf (Kim *et al.*, 2010), rock-climbing (Hodgson *et al.*, 2009), and resistance training (Leite *et al.*, 2011; Rahimi *et al.*, 2010b; Shariat *et al.*, 2012). However there is conflicting evidence regarding the salivary cortisol response. There was no change observed following a professional soccer match (Moreira *et al.*, 2009b), high power resistance exercise (Fry and Lohnes 2010), alongside no change following repeated high intensity sprints of 200 metres over two hours (Sinaei *et al.*, 2012). This definitively identifies cortisol secretion as intensity and duration dependent which was first identified in subjects who ran at various intensities and durations which significantly affected cortisol secretion (Kindermann *et al.*, 1982). Rugby union has also been demonstrated to increase salivary cortisol. Significant increases were observed following an international rugby game that remained elevated above pre-game levels 14 hours later (Cunniffe *et al.*, 2010). Potentially related to the state of recovery, they suggest salivary cortisol may be an effective indicator of over-training when combined with testosterone. In contrast, Elloumi *et al.* (2003)

observed a two and a half fold salivary cortisol increase following an international rugby game with levels returning to baseline within four hours. This may be attributed to the level of competition, the inter-individual variability that is so common and often neglected, or the type of sample collection and analysis.

Meanwhile, salivary cortisol has also been utilised to identify athletes in a state of fatigue or over-training. Significant elevations in salivary cortisol were observed during periods of heavy training in elite swimmers compared to controls. This corresponded with over-reaching and training and correlated ( $r = 0.5$ ) with a depressed mood state (O'Connor *et al.*, 1989). A study using 10 under-performing athletes identified a significantly higher cortisol response than those with over-training syndrome than those with non-functional over-reaching following two exercise bouts (Meeusen *et al.*, 2010). Similar observations were seen in elite volleyball players (Naghibi *et al.*, 2013) and soccer players (Amiri *et al.*, 2011). However an intense period of training or competition does not always result in increased salivary cortisol. Olympic weightlifters completing a rigorous five week training protocol that included 100 - 200 sets per week lifting, showed no change. However a significantly elevated cortisol concentration was identified during competition that corresponded to a better 1-RM (Crewther *et al.*, 2011). Similarly, completing 10 marathons in 10 days also identified no changes in baseline salivary cortisol concentrations (Southworth *et al.*, 2013). In a rugby context, significant increases were observed following a game that remained elevated for 36 hours. In conjunction with testosterone, cortisol provided information that players were in a catabolic state up to five days post-game (West *et al.*, 2014).

Further rugby based evidence for salivary cortisol measurements have identified it as a sensitive marker of anxiety that may be related to a feeling of dominance or submissiveness. A study identified professional rugby players had elevated salivary cortisol levels that corresponded with venue and opposition (Morgan 2011). A similar pattern in field hockey was identified where higher cortisol concentrations were observed during home ground games (Carré *et al.*, 2006). This type of theory is validated by increased levels of cortisol and alpha-amylase in those with the greatest feelings of perceived dominance (Kivlighan and Granger 2006) or with the most aggression (Gordis *et al.*, 2006). Additionally, salivary cortisol has also been shown to correlate ( $r = 0.39$ ,  $p < 0.05$ ) with neuromuscular power in rugby players (Crewther *et al.*, 2009), correspond to travel fatigue (Bullock *et al.*, 2009), and

show potential as an early indicator of successfully executed performances in competitive rugby union following mid-week training and analysis (Gaviglio and Cook 2014). This could potentially identify simple measurements of cortisol or testosterone in predicting performance, however considerably more evidence is required based on contrasting research in power-lifters whose cortisol concentrations were not related to performance (Le Panse *et al.*, 2010).

## 1.7 OXIDATIVE STRESS

The term oxidative stress is applied to a particular state where there is a serious imbalance between production of reactive species and antioxidant defence (Halliwell and Whiteman 2004). However, in a relatively recent review (Il'yasova *et al.*, 2012), the term “oxidative status” may be applicable as a result of the large inter-individual variation in oxidative stress biomarkers (Keaney *et al.*, 2003) and the lack of a “normal” reference range. This variation can be attributed to genetic and epi-genetic differences (Lacy *et al.*, 2000), endogenous promoters of ROS, and chronic inflammation (Perwez Hussain and Harris 2007). Regardless, ROS are produced on a continual basis in aerobic organisms through cellular respiration and antibacterial defence among others (Halliwell and Gutteridge 2007); with the relationship between antioxidant defence capacities and their production controlling the level of associated oxidative damage.

Anti-oxidant defence mechanisms include enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidases, sequesters of metal ions, and endogenous antioxidants such as glutathione, ubiquinol, bilirubin, uric acid,  $\alpha$ -tocopherol, and ascorbic acid. They work in tandem to eliminate or “control” the vast array of ROS produced which includes superoxide, hydrogen peroxide ( $H_2O_2$ ), hydroxyl radicals, singlet  $O_2$ , NO, peroxynitrite, hyperchlorite and other secondary radical species. Their generation of course increases following any aerobically based exercise and contracting skeletal muscle which can overwhelm the anti-oxidant defence capacity resulting in oxidative damage to cellular constituents (Ji 1999; Powers and Jackson 2008).

The direct measurement of oxidative stress in humans cannot be conducted due to the short half-life of ROS (Halliwell and Gutteridge 2007). It is therefore recognized that the products of non-enzymatically derived compounds of oxidative stress provides a direct index of the extent of oxidative modifications produced by ROS (Il'yasova *et al.*, 2012). A marker (summarised in Fig. 1.4) of oxidative stress has to have four characteristics:

- 1) Chemically unique and detectable.
- 2) Increased or decreased during periods of oxidative stress.
- 3) Possess relatively long half-lives.
- 4) Not impacted by other cellular processes (Halliwell and Gutteridge 2007).

This is particularly true for urinary biomarkers of oxidative damage; however plasma concentrations of anti-oxidant enzymes can also be used to provide a quantitative evaluation. Superoxide dismutase for example has been shown to increase in activity from 20 – 112 % following endurance based training (Powers and Jackson 2008). Examples of oxidatively modified molecules include F<sub>2</sub>-isoprostanes formed through arachidonic acid oxidation (Morrow *et al.*, 1990), 8-hydroxy-2'-deoxyguanosine, a product of DNA oxidation (Kadiiska *et al.*, 2005), malondialdehyde, a toxic ketone formed through peroxidation of polyunsaturated fatty acids, and dityrosine, formed through protein oxidation (Halliwell and Whiteman 2004). However urinary biomarkers have the added advantages due to lower levels of material that can be oxidized, paralleled with lower levels of ROS promoters. Therefore this process minimizes the chance of artificial oxidation and chances of false-readings.

### 2.7.1 Exercise-Induced Oxidative Stress

Intense exercise is commonly associated with an increase in ROS (Ji 1999). This was first determined by Davies (1982b) who identified contracting skeletal muscle produced ROS that was potentially damaging to tissue. Exceeding the anti-oxidant defence capacity can result in oxidative alteration to lipids, DNA and proteins (Roebuck 1999), induce muscle damage (Duarte *et al.*, 1993), and contribute to the effect of delayed onset muscle soreness and fatigue (Aoi *et al.*, 2004). The exercise-induced increases of O<sub>2</sub> consumption, ischemia-reperfusion phenomena, and inflammation are recognized as the major sources of ROS

during and after physical exercise (Alessio 1993; Powers and Lennon 1999; Sjödin *et al.*, 1990). When muscle damage occurs simultaneously, a secondary wave of ROS is produced through the abundance of inflammatory cells invading the area.

### Markers of oxidative stress

<p><b><u>Oxidants</u></b></p> <p>Superoxide anions Hydroxyl radical Hydrogen peroxide Peroxynitrite Other radicals</p>	<p><b><u>Antioxidants</u></b></p> <p>Glutathione Ascorbate Alpa-tocopherol Total antioxidant capacity</p>
<p><b><u>Oxidation products</u></b></p> <p>Protein carbonyls Isoprostanes Nitrotyrosine 8-OH-dG 4-Hydroxy-nonenal Malondialdehyde</p>	<p><b><u>Antioxidant/Pro-oxidant balance</u></b></p> <p>GSH/GSSH ratio Cysteine redox state Thiol/disulfide state Other?</p>

**Figure 1.4.** The four broad classes of biomarkers used to assess cellular oxidative stress in tissues. These include oxidants, products of oxidation, levels of enzymatic antioxidants and the cellular redox balance. Reproduced with permission as part of Powers *et al.* 2008.

Contracting muscle typically produces superoxide at a number of cellular sites. Initially, the most predominant of these sites included Complex I and III of the electron transport chain of the mitochondria (Barja 1999; Muller *et al.*, 2004) with two to five percent of the total O<sub>2</sub> consumption thought to undergo a reduction to form superoxide (Boveris and Chance 1973; Loschen *et al.*, 1974). However more recent evidence shows mitochondria are in a state that does not allow for the production of ROS (Di Meo and Venditti 2001) with only ~ 0.15 % of the O<sub>2</sub> consumed being converted into superoxide (St-Pierre *et al.*, 2002). Other locations of oxidant production include nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymes of the sarcoplasmic reticulum (Xia *et al.*, 2003) and transverse tubules (Espinosa *et*

*al.*, 2006), as well as NO synthases shown to increase NO production during muscle contraction (Roberts *et al.*, 1999). Meanwhile, oxidant production increases following the direct damage to muscle. The invasion of phagocytic cells including macrophages and neutrophils known to produce a vast array of oxidants (Peake 2002) during their part of tissue regeneration can contribute to the total oxidative stress (Forner *et al.*, 1994; Ortega *et al.*, 1993; Smith *et al.*, 1996). Following exercise, both neutrophils and macrophages have been shown to invade an area of damage (Müns 1994) resulting in an increased flux of oxidants that is also differentially affected by the duration and intensity of the activity (Pyne *et al.*, 2000).

There is an abundance of research examining the effects of various exercise on changes in oxidative status. However it is known that physical exercise increases the anti-oxidant defence capacities in order to reduce the effects of ROS (Miyazaki *et al.*, 2001). For extensive reviews, see Powers *et al.* 2008, Finaud *et al.* 2006 and Fisher-Wellman *et al.* 2009. Strenuous exercise may have the opposite effect. Specifically, during a 21 km run completed as fast as possible, subjects were found to have significant increases in malondialdehyde as well as significant increases in CK and myoglobin. While this provides information pertaining to muscle damage and oxidative stress, it found no muscle damage at 24 hours post-race through ultra-structural examination. This indicates the release of CK and myoglobin may be the result of free radical induced cell membrane damage rather than mechanical muscle damage (Goodman *et al.*, 1997).

Significant increases in SOD, catalase, and glutathione peroxidase activities have also been observed in lymphocytes following high intensity interval training (Fisher *et al.*, 2011), while intense treadmill running for 60 minutes resulted in observed increases in total lymphocyte protein carbonyls, plasma lipid peroxides, total antioxidant capacity, and intracellular thiol content (Turner *et al.*, 2011). Similarly, anti-oxidant enzyme activities have been shown to decrease following both submaximal and maximal cycling (Berzosa *et al.*, 2011), while resistance exercise and soccer significantly increase thiobarbituric acid reactive substances (TBARS), protein carbonyls and decreases antioxidant activities (non-protein sulfhydryl, SOD, catalase) (Cardoso *et al.*, 2012; Fatouros *et al.*, 2010). These processes implicate oxidative stress as a surrogate for over-training assessment in professional athletes (Finaud *et al.*, 2006b; Palazzetti *et al.*, 2003; Tiidus 1998). Tanskanen *et al.* (2010) identified

significantly higher levels of plasma protein carbonyls in over-trained athletes compared to controls at baseline. An increase in O<sub>2</sub> radical absorbance capacity and malondialdehyde at baseline and after six months recovery following exercise to exhaustion in the control group only was also observed. Moreover, the cellular redox balance following a soccer game continually declined post-game for up to 72 hours which may provide more evidence for post-exercise recovery management (Fatouros *et al.*, 2010).

Impact related sport shows similar properties. A study identified an increase in conjugated diene oxidation and a decrease in Vitamin E production during periods of intense training in professional rugby (Finaud *et al.*, 2006b). This can be contrasted with a study that identified a slightly improved lipoprotein profile and enhanced anti-oxidative status in 15 well-trained rugby players compared to sedentary controls (Evelson *et al.*, 2002). However the immediate post-game effect of contact sports on oxidative status shows increases in low density lipoprotein (LDL) oxidation, TBARS and conjugated dienes following a rugby game which also identified oxidative stress adaptations in trained vs. untrained players (Chang *et al.*, 2002). Similarly a study on underwater rugby identified significant increases in antioxidant enzymes (Cavas 2005), while American Football seems to alter serum peroxide concentrations on a very individual basis during a competitive season (Schippinger *et al.*, 2002).

### 1.7.2 Neopterin, Biopterin, Xanthopterin and Myoglobin

Neopterin as mentioned (see section 1.12.2), is an oxidation product of 7,8-dihydroneopterin which is produced primarily from IFN- $\gamma$  activated macrophages. The measurement of NP in urine or plasma has been typically used as a marker of inflammation, however the vast majority of the literature fails to incorporate the quantification of 7,8-dihydroneopterin to give total macrophage activation and therefore a true understanding of the inflammatory process. Therefore when research measures NP alone, it is providing a measurement of the total level of oxidative stress associated with a disease or type of exercise. As mentioned, hypochlorite is the only known oxidant capable of converting 7,8-dihydroneopterin to NP *in vivo*. By measuring changes in urinary NP as indicated previously, an understanding of the total hypochlorite present and oxidative stress is gathered. Similarly, xanthopterin (XP), which is also an oxidation product of 7,8-dihydroneopterin, provides another alternative for

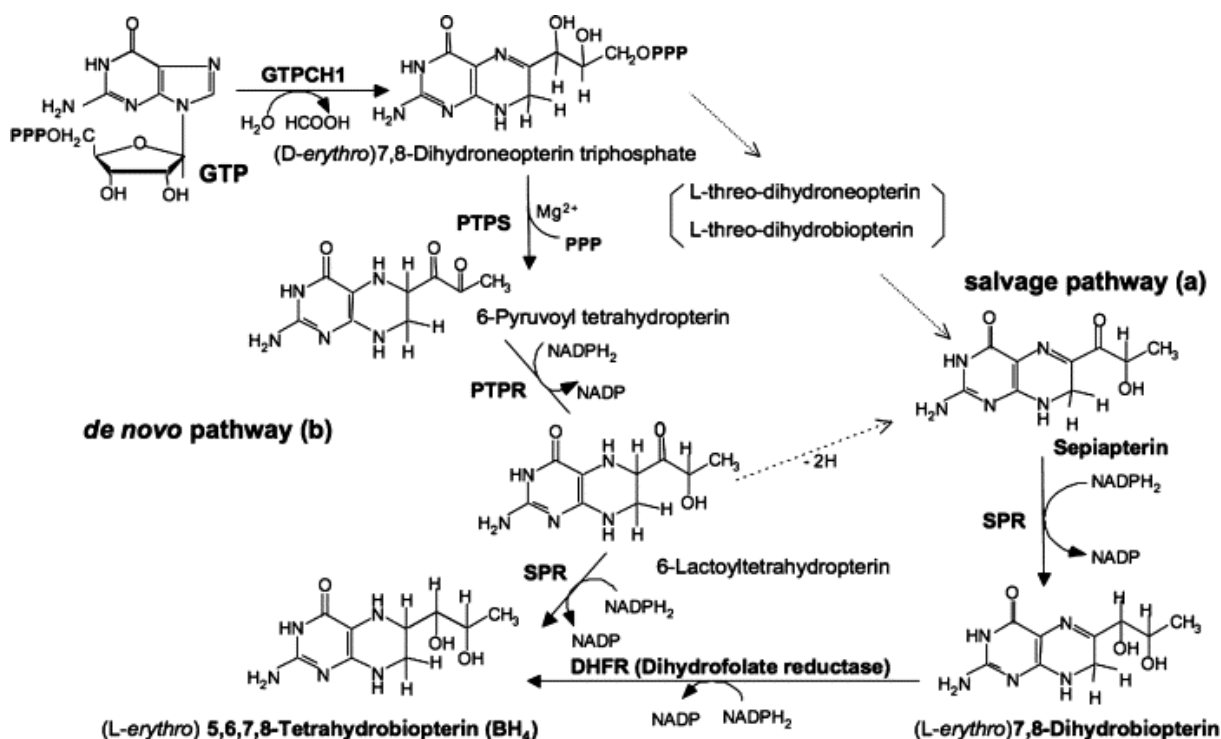


quantification of oxidative stress. Xanthopterin is the final oxidation product of both 7,8-dihydroneopterin and 7,8-dihydroxanthopterin. Oxidation of 7,8-dihydroneopterin with  $\text{H}_2\text{O}_2$  results in the formation of 7,8-dihydroxanthopterin at very high yield (90%) which can then oxidize further to XP (Laura Dántola *et al.*, 2008). Furthermore, dihydroxanthopterin also happens to be the main oxidation product of 7,8-dihydrobiopterin ( $\text{BH}_2$ ) which can also be oxidized to biopterin (BP). Xanthopterin has been used clinically to assess differences between cancer patients and controls (Gamagedara *et al.*, 2011; Kośliński *et al.*, 2011) and diagnosing those with liver disease and hemolysis. Its detection following exercise has yet to be explored which may provide useful additional information to the abundantly used pteridines in disease and exercise research.

Biopterin is the final oxidation product of  $\text{BH}_2$  which in turn is the oxidation product of tetrahydrobiopterin ( $\text{BH}_4$ ) in several cell types (Werner *et al.*, 1989). Quantifying the change in BP can provide further information about the total level of oxidative stress associated with exercise. Tetrahydrobiopterin is synthesized in two separate pathways: *de novo* and salvage (Fig. 1.5). The *de novo* pathway is a  $\text{Mg}^{2+}$ ,  $\text{Zn}^{2+}$  and NADPH-dependent reaction from GTP via 7,8-dihydroneopterin triphosphate, 6-pyruvoyl-5,6,7,8-tetrahydropterin involving GTPCH1, 6-pyruvoyl-tetra-hydropterin synthase and sepiapterin reductase (Thony *et al.*, 2000). The salvage pathway involves the NADPH-dependent enzyme  $\text{BH}_2$  reductase to convert  $\text{BH}_2$  back to  $\text{BH}_4$  following its production with  $\text{H}_2\text{O}$  during the synthesis of the monoamine neurotransmitters (Pawlina and Ross 2007).

Tetrahydrobiopterin has been primarily investigated for its action as a natural co-factor of the aromatic amino acid hydroxylases used in the degradation of the amino acid phenylalanine and in the biosynthesis of the monoamine neurotransmitters. It is also used in all three forms of NO synthase with specific cellular functions including cell proliferation and growth (Tanaka *et al.*, 1989). Tetrahydrobiopterin is also a free-radical scavenger (Shimizu *et al.*, 1998) whose deficiency has been attributed to a reduction in NO production and associated vasodilation (Bagi *et al.*, 2004). The quantification of  $\text{BH}_4$  following exercise has been widely neglected. However post-exercise increases in  $\text{BH}_4$  and total BP have been observed following exhaustive cycle ergometry. Changes were intensity dependent that only increased in those subjects who cycled to complete exhaustion (Mizutani *et al.*, 1994). Indirect measurements of  $\text{BH}_4$  production can be linked to changes in adrenaline, dopamine or NO

following exercise. Furthermore, it is recognised that intense exercise results in a marked increase in production of several monoamine neurotransmitters and NO (Bortz 2nd *et al.*, 1981; Stamler and Meissner 2001) which signifies its role in vasodilation, reward-motivated behaviour and the fight or flight response (Daubner *et al.*, 2011; Kwon *et al.*, 1990). A study investigating the effect of NO observed an attenuation of acetylcholine activation of endothelium vasodilation when an inhibitor was used (Goto *et al.*, 2003). The release of adrenaline and nor-adrenaline is intensity dependent. A study investigated the secretion in a cohort of males running at intensities above and below their lactate threshold (Pritzlaff *et al.*, 2000) as well as being attenuated by the production of lactate during exercise (Fattor *et al.*, 2005). Furthermore, the release of adrenaline and nor-adrenaline significantly increase following short repetitive intense exercise (10x6s cycle sprints) while dopamine does not (Bracken *et al.*, 2009). In contrast running on a treadmill for 30 minutes at an average speed of 8.7 km/h resulted in no change in synaptic dopamine concentration in the brain after a PET scan (Wang *et al.*, 2000).



**Figure 1.5.** Biosynthetic pathways of tetrahydrobiopterin: via the salvage pathway through the intermediates sepiapterin and BH<sub>2</sub> (a), and the de novo pathway (b). GTPCH1; GTP

cyclohydrolase I, PTPS; 6-pyruvyl-tetrahydropterin synthase, SPR; sepiapterin reductase, DHFR; dihydrofolate reductase. Reproduced with permission as part of Hasegawa et al. 2005.

Biopterin however, the oxidation product of  $BH_2$  has been shown to be elevated in conjunction with NP in the urine of children with autistic-like-disorders (Harrison and Pheasant 1995), such as autism (Messahel *et al.*, 1998) and unipolar depression (Duch *et al.*, 1984). However the majority of the studies measure  $BH_4$  alone (Eto *et al.*, 1992) or total BP and neglect the significance of BP. It becomes difficult to ascertain what measurements are being conducted in some studies especially when some compare “BP” with  $BH_4$  concentrations. In an exercise and oxidative stress context, the elevation in total BP ( $BP + BH_2 + BH_4$ ) and BP alone can provide an indirect measurement of exercise related stress as a result of  $BH_4$ 's involvement as a cofactor for various reactions (NO synthetase and the aromatic amino acid hydroxylases) (Kaufman 1986) and the involvement of ROS in the oxidation to BP. The quantification of BP during exercise has not been measured as a source of oxidative stress. It may provide a simple additional source of exercise-induced oxidative status in a non-invasive manner. Meanwhile the simultaneous measurement of BP and NP provides two equally important pieces of information whilst keeping assay costs at a minimum.

Heme proteins such as myoglobin have also been postulated as markers of oxidative stress in the urine of rhabdomyolysis patients (Reeder *et al.*, 2002). Their “rogue” enzymatic activity is sequestered by cell membranes, however in the event of its integrity loss, they can escape causing a cascade of oxidation events that lead to lipid peroxidation (Rogers *et al.*, 1995). The reaction occurs through the tyrosine peroxyl radical of the iron containing porphyrin ring which has been shown to initiate myoglobin induced lipid peroxidation directly (Giulivi and Cadenas 1998; Reeder *et al.*, 2002) or by releasing free ferrous ions which could generate hydroxyl radicals through the Fenton reaction (Rice-Evans *et al.*, 1993). A direct interaction between myoglobin and the mitochondria in relation to ARF was identified which may give rise to this iron release with subsequent oxidation of the mitochondrial membrane (Plotnikov *et al.*, 2009).

Oxidation has been demonstrated as the principal causative factor in rhabdomyolysis-induced renal failure as it undergoes auto-oxidation to the ferric form promoting lipid peroxidation

through the Feric/Ferryl iron ( $\text{Fe}^{3+}/\text{Fe}^{4+}$ ) redox cycle (Giulivi and Cadenas 1998). This cycling has been revealed to elevate urinary isoprostane concentrations in animal models 7.3-fold above controls through its accumulation in the kidney (Moore *et al.*, 1998). Further studies have also shown myoglobin to increase ROS and lipid peroxidation (Shah and Walker 1988; Zager 1996a).

Heme initiated oxidative stress is possible when ultra-structural damage to the muscle membrane results in leakage of intracellular proteins (Brown and Hill 1991). In a rugby context, force related impacts are the most likely cause of this damage (Smart *et al.*, 2008) and it is possible there is a relationship between the elevation in myoglobin and markers of oxidative stress. Furthermore, greater oxidative damage may arise from the formation of heme-protein cross link species (Mb-H) as a result of  $\text{H}_2\text{O}_2$  oxidation of Mb (King and Winfield 1963). The formation of this stable product *in vitro* (Catalano *et al.*, 1989), *in vivo* (Holt *et al.*, 1999) and its detection in the urine of patients with rhabdomyolysis (Holt *et al.*, 1999) identifies its oxidative potential in conjunction with native myoglobin. This reaction has been shown to enhance LDL and NADH oxidation compared to native Mb (Vuletic *et al.*, 2000) with only small concentrations of  $\text{H}_2\text{O}_2$  required for its oxidation into a redox active enzyme (Osawa and Korzekwa 1991). Oxidized LDL is considered one of the principal causative factors in the formation of atherosclerotic plaques (Hegyi *et al.*, 1996; Lassila 1993). The formation of other heme-protein cross-linked species including hemeoglobin (Osawa *et al.*, 1993) and liver microsomal P450 cytochromes (Osawa and Pohl 1989) following  $\text{H}_2\text{O}_2$  oxidation may enhance the level of lipid peroxidation catalysis by these adducts that could significantly influence exercise-induced oxidative stress in impact related sports. In contrast, myoglobin's instability may cause loss of its oxidative potential due to factors such as temperature, pH, unidentified urinary compounds smaller than 10kDa, and time to analysis (Chen-Levy *et al.*, 2005), whilst its extremely fast elimination kinetics (Suzuki *et al.*, 1999) may also contribute to its inability to act as an oxidizing agent.

## 1.8 CARDIOVASCULAR STRESS

Exercise-induced cardiovascular stress is a broad term given to the effect of external stimuli on the mechanistic operations of the heart and its ability to function optimally. Typically this stimuli is associated with hemodynamic changes that alter the loading conditions of the heart

through alteration in stroke frequency and volume to provide working muscles with an increased supply of O<sub>2</sub> and nutrients (Fagard 1997). The result of continuous exercise training results in morphological changes, including increases in left ventricular chamber size, wall thickness, and mass (Pluim *et al.*, 2000). For example, those that perform endurance exercise such as ultra-endurance adventure races or cycling competitions covering more than 3000 kilometres per week develop predominantly increased left ventricular chamber size with a proportional increase in wall thickness. This is caused by volume overload associated with the high cardiac output of endurance training (Pluim *et al.*, 2000). Athletes involved in static or isometric exercises including weightlifting, develop predominantly increased left ventricular wall thickness with unchanged left ventricular chamber size. This is caused by pressure overload accompanying the high systemic arterial pressure found in this type of exercise (Pluim *et al.*, 2000).

The exercise that induces these morphological changes is subsequently accompanied by excess strain of the heart that results in both reversible and irreversible myocardial injury. In a professional context, the control of this cardiac stress is an essential tool for monitoring athlete health and recovery. Even though exercise normally results in reversible myocardial injury, sub-clinical myocardial cell necrosis or damage is a possibility (Ohba *et al.*, 2001). However there is evidence of reversible injury in the cardiomyocytes in healthy athletes by the normalisation of cardiac troponins 24 hours post-exercise (Scharhag *et al.*, 2005; Siegel *et al.*, 2001). This suggests that increases in heart stress biomarkers may be normal in healthy athletes and are without a pathophysiological significance (Koller 2003). It is implied that athletes with a sustained increase in selected heart stress markers undergo further cardiological tests (Herrmann *et al.*, 2003; Urhausen *et al.*, 2004), especially when on the rare occasion an athlete can suffer from myocardial infarction even in the absence of coronary heart disease and hypertrophic cardiomyopathy (O'Keefe *et al.*, 2012; Patil *et al.*, 2012)

Heart damage analysis has been assessed through the availability of specific cardiac biomarkers (Scharhag *et al.*, 2004; Shave *et al.*, 2007), heart rate monitors (Jeukendrup and Diemen 1998), heart rate variability (Carter *et al.*, 2003), echocardiography (ECG) (Vidotto *et al.*, 2005) and other imaging techniques (Breuckmann *et al.*, 2009). Cardiac troponins (cTn) have been widely used for assessment of myocardial damage following strenuous exercise. Immediate post-marathon increases in cardiac troponin T (cTnT) have been

observed that returned to baseline within 72 hours (Scherr *et al.*, 2011), while monitoring of 37 young adolescents between the ages of 13 and 17 following a marathon identified significant elevations of both cTnT and cardiac troponin I (cTnI). For five of the subjects, their values exceeded the reference values for myocardial infarction but all returned below the limit 24 hours later (Traiperm *et al.*, 2012). Similarly, ECG during a marathon identified changes in heart variability and T-wave alternans that can be attributed to a physiological process (Franco *et al.*, 2014), while magnetic imaging has identified the development of coronary plaque on the hearts of endurance athletes that can cause atrial fibrillation (La Gerche *et al.*, 2012; Möhlenkamp *et al.*, 2008).

The long-term effects of endurance and high intensity exercise may result in later life arrhythmias or development of scattered fibrosis and scarring in the walls of the atria, inter-ventricular septum and right ventricle. This is commonly seen in experienced marathon runners (Breuckmann *et al.*, 2009). The endurance aspect coupled with high force impacts of rugby union which have sporadically been shown to cause cardiac damage and associated stress may doubly influence cardiac stress in a sport that is so common. As an example, rats that were inflicted with blunt trauma to the chest resulted in some form of cardiac arrhythmia with cardiac injury present in 31% of the cases (Wang *et al.*, 2003), while meta-analysis reviews of chest trauma in humans identified overall thoracic morbidity rate at 36% and mortality rate at 15.5% (Shorr *et al.*, 1987) following blunt force trauma. It is evidently clear non-penetrating trauma to the chest can cause severe cardiac stress as seen in a young man who died at the scene of a blunt force chest trauma that resulted in the detachment of the coronary artery (Darok *et al.*, 2001). When coupled with the endurance aspect of contact sports, total cardiac stress may lead to both physiological and pathological changes.

### 1.8.1 N-Terminal Prohormone of Brain Natriuretic Peptide

N-Terminal prohormone of brain natriuretic peptide (NT-proBNP) is the cleaved inactive fragment of brain natriuretic peptide (BNP) that was first identified in 1988 (Sudoh *et al.*, 1988). It is synthesized by cardiac myocytes (Hall 2004) and fibroblasts (Tsuruda *et al.*, 2002). Secreted primarily by cardiac myocytes in response to ventricular wall tension or stress (Maggia *et al.*, 1994; Maisel *et al.*, 2001) in a paracrine or endocrine fashion (Wu *et al.*, 1996), its secretion is dependent on activation of the BNP gene from atrial stress due to the

small amounts found in the atrial granules compared to atrial natriuretic peptide (Hall 2004). Once secreted, NT-proBNP has several functions that ultimately reduce myocardial wall stress. These include natriuresis, vasodilation, and a sympatho-inhibitory effect (Brunner-La Rocca *et al.*, 2001; Burnett Jr 1999).

NT-proBNP has been shown to rise in patients with cardiac dysfunction (Lubien *et al.*, 2002), chronic heart failure (Maisel 2001), pulmonary embolism (Tulevski *et al.*, 2001), myocardial infarction (Sumida *et al.*, 1995) and healthy individuals after strenuous endurance exercise (Corsetti *et al.*, 2012; Herrmann *et al.*, 2003). It has been suggested as a reliable and sensitive marker of cardiovascular stress following exercise that also has potential cytoprotective and growth regulating effects (Banfi *et al.*, 2010; Scharhag *et al.*, 2005). Its sensitivity and specificity also allow for simple quantification in serum and urine with values exceeding 125 pg/mL considered above the pathological threshold (Banfi *et al.*, 2010). With an extended half-life of 60 - 120 minutes in comparison to the 20 minutes of BNP (Faviou *et al.*, 2008), comparable resistance to neutral endopeptidase (Smith *et al.*, 2000), and unhindered urinary excretion, quantification of NT-proBNP by ELISA is relatively easy based on its first detection by Hunt *et al.* (Hunt *et al.*, 1995).

NT-proBNP as a marker of cardiovascular stress remains controversial. Some studies have identified a simultaneous rise in cTnT which was attributed to myocardial wall necrosis (Rifai *et al.*, 1999). Other studies have not identified any simultaneous rise (Konig *et al.*, 2003; Scharhag *et al.*, 2005) which suggests no subclinical myocardial lesion. This is further corroborated by two studies on marathon competitors who observed increases in both NT-proBNP and cTnI which also resulted in no myocardial damage as screened by ECG (Scharhag *et al.*, 2006). The release of NT-proBNP without irreversible injury, lesion or necrosis is potentially due to the transitory increased membrane permeability following intense exercise (Scharhag *et al.*, 2005). A significant increase in NT-proBNP was observed that returned to pre-race levels 72 hours later. It was accompanied by altered cardiac myocyte metabolism rather than necrosis (Scherr *et al.*, 2011). This hypothesis seems more likely due to the large number of studies using imaging techniques following strenuous exercise of various varieties that result in an increase in both NT-proBNP and cardiac troponins without any permanent damage. However the detection of a substantial increase either immediately before or in the days following warrants further exploration for possible injury.

NT-proBNP has been used to assess cardiovascular stress in endurance-based running (Mattsson *et al.*, 2010), cycling (Corsetti *et al.*, 2012), triathlon (Leetmaa *et al.*, 2008), military training (Schmidt 2011), rugby training (Banfi *et al.*, 2008), resistance training and indoor soccer (Carranza-García *et al.*, 2011). Unfortunately the effect of impact related trauma has not been assessed on the secretion of NT-proBNP. Significant increases were observed immediately post and at 24 hours post a five to six day non-stop endurance race of (Mattsson *et al.*, 2010). Similar changes have also been observed following a 160 kilometre ultra-marathon (Scott *et al.*, 2009), 100 kilometre ultra-marathon (Ohba *et al.*, 2001), a standard marathon (Scherr *et al.*, 2011), and high intensity rugby interval training (Banfi *et al.*, 2008). Additionally its secretion is independent of intensity but dependent on duration when subjects were asked to run for 45, 90 and 180 minutes at 85% and 95%  $\dot{V}O_2\text{max}$  (Serrano-Ostáriz *et al.*, 2011). More evidence may have to be provided about intensity differences based on the values chosen in this study. One of the most compelling studies to date was that of professional road cyclists in the Giro d'Italia. NT-proBNP was measured throughout the course of the 22 day cycle race with concentrations continuously increasing throughout. This myocardial stress overload may result in arrhythmias in later life as discussed, or a decrease in performance throughout the race (Corsetti *et al.*, 2012).

Furthermore, there is equivocal evidence surrounding the effect of training and adaptation on the secretion of NT-proBNP. With its cytoprotective and wall tension reducing properties, it could be assumed some form of training may alter the secretion or permeability of the myocyte walls in response to an exercise stress. Minor increases in NT-proBNP have been observed in athletes compared to controls in response to exercise bouts. This could be attributed to higher training volumes and higher left-ventricular mass (Scharhag *et al.*, 2004). This also provides further evidence of the cyto-protective properties of NT-proBNP which may regulate myocardial adaptation in healthy athletes. Similarly amateur athletes who train less than 35 miles per week in preparation for a marathon had the most marked abnormalities in cardiac structure, function and biomarkers compared to those who trained more than 35 miles per week (Neilan *et al.*, 2006). Equivocal evidence also surrounds the baseline secretion of NT-proBNP between trained and untrained individuals. While some say there is no difference (Scharhag *et al.*, 2004), others identify significant differences most likely a result of “athlete’s heart” (Banfi *et al.*, 2010), and some show combined endurance and



resistance training reduces NT-proBNP secretion in patients with chronic heart failure (Conraads *et al.*, 2004).

## 1.9 LUNG DAMAGE

Similar to inflammation in response to trauma elsewhere in the body, damage to the lung results in an influx of various inflammatory and immune cells (and associated events). This can lead to structural damage in the airways and parenchyma in severe cases. In a physiological context, direct damage to the lung from trauma or a disease based inflammatory reaction (cystic fibrosis) can result in substantial loss of oxygenation and complications can arise. From an exercise-based perspective, lung injury greatly impairs breathing and reduces alveolar gas exchange. This subsequently decreases delivery of O<sub>2</sub> and removal of carbon dioxide (CO<sub>2</sub>) to the working muscles which can significantly affect performance.

The physical stress encountered in a single game of rugby is associated with a high risk of injury (Kaplan *et al.*, 2008) as a result of larger, faster and stronger players, large tackle forces (Usman *et al.*, 2011), and the onset of the professional era that is designed to increase speed of play. Players can average as much as 6715 running metres (Coughlan *et al.*, 2011) which includes work to rest ratios as low as one to five in a game (Austin *et al.*, 2011). The intermittent nature of rugby union, other contact sports and endurance based competition can cause substantial respiratory stress. Expiratory flow limitation has been demonstrated to occur more often in exhaustion of females than males during an incremental cycle test. This suggests that women utilise a greater majority of their ventilatory reserve compared to men (Guenette *et al.*, 2007). This has been similarly reported in other studies (Johnson *et al.*, 1992; McClaran *et al.*, 1998), where it may cause reflex inhibition of the hyper-ventilatory response or an alteration in operational lung volumes. This research also identified that elite athletes reach their mechanical limits of the lung and respiratory muscle for producing alveolar ventilation during maximal exercise (Johnson *et al.*, 1992). Resistive training studies have reported increases in maximum inspiratory pressure in the range of eight to 45 % (Sonetti *et al.*, 2001), while a study using proportional assist ventilation observed significant decreases in diaphragmatic muscular fatigue during cycling exercise (Babcock *et al.*, 2002). Respiratory stress following exercise does not always occur however. Younes and Kivinen (1984) noticed that lung elastic recoil is unchanged during maximal exercise. Additionally inspiratory muscles were found to operate within a potentially fatiguing range, however

maximum ventilation was not continued for a sustained period to induce mechanical injury. In relation to the intermittent nature of rugby union, this may be applicable. In conjunction with breathing frequencies as high as 62 breaths per minute and tidal volumes rising as high as  $3.29 \text{ L}^{-1}$  during maximal exercise (Clark *et al.*, 1983), respiratory mechanics assessment for lung injury and inflammation is an important aspect in a player's overall health.

Meanwhile, one of the major body parts at risk of contact injury is the trunk (Kaplan *et al.*, 2008). Blunt chest trauma in severe cases is known to cause myocardial contusion (Pai 2014), while less severe injuries include hematoma, bruises and fracture of the ribs or sternum that can directly cause a life threatening lesion. While the impact forces of rugby tackles are not comparable to that of crush victims or car crash accidents, the repeated impacts in conjunction with moderate forces may seriously complicate the respiratory mechanics and the player's ability to compete maximally.

Visible damage is easily diagnosed through simple physical examination and other biological markers; however assessment of the extent of any internal injuries cannot be made through superficial examination. For example there may be changes to respiratory mechanics through lung injuries occurring due to player collisions around the trunk region during a game (Borghi-Silva *et al.*, 2008) which can be difficult to diagnose. With as many as 166 tackles per hour in the modern game (McIntosh *et al.*, 2010), there is a risk of changes in respiratory mechanics due to lung injury; however these cannot currently be identified without the use of detailed radiographic imaging (Hayashi *et al.*, 2013). Given the highly invasive nature of such techniques, the assessment of lung injury is often neglected because of risk of radiation exposure, a dearth of non-invasive alternatives and perhaps due to the belief that such injuries are of relatively minor medical significance (Schmidt 2012). This negligence potentially reduces subsequent rugby player performance and recovery, and potentially exposes them to the risk of long term harm if they are poorly monitored (Smith 2011).

### 1.9.1 Ventilation and Elastance

Critically ill patients are assessed through specialised protocols (Oostveen *et al.*, 2003) or model-based methods (Steimle *et al.*, 2011). Model-based methods are patient-specific approaches that offer the ability to identify intra- and inter-patient variability that are

becoming increasingly popular as they provide a unique insight of a patient's condition and response to treatment without the need of specialised or additionally invasive protocols (Chase *et al.*, 2011; Sundaresan and Chase 2012). They are based on selecting an optimal positive end-expiratory pressure (PEEP) to maximise patients' lung recruitment, prevent alveoli collapse, and avoid ventilator induced lung injury (Gattinoni *et al.*, 2010). Currently nothing is known about whether these model-based methods can be used to assess the respiratory mechanics of healthy individuals and the assessment of lung injury following exercise.

In the case of the critically ill, patient-specific respiratory mechanics are assessed through information gathered by a ventilator to aid clinical decision making (Chase *et al.*, 2011). Ventilators are sophisticated and sensitive devices designed to provide breathing support to critically ill patients. Ventilators have the inbuilt capability to capture high frequency, accurate, patient specific pressure and flow information (Chiew *et al.*, 2011). The commonly used spirometer for assessing lung function measures only flow information. Use of a ventilator to capture athlete specific respiratory mechanics information has the potential to allow for effective and non-invasive assessment of an athlete's respiratory condition.

Elastance is a measure of the tendency of a hollow organ to recoil toward its original dimensions upon removal of a distending or compressing force (Dictionaries 2007). Dynamic lung elastance ( $E_{drs}$ ) is a time-variant lung elastance during each breath in mechanical ventilation which has been shown to be a sensitive measurement of overall physiological condition than a first order model using constant lung elastance. There is no research on the effects of exercise on lung elastance as a measure of respiratory mechanics. Potential lung injury or inflammation occurrence due to a severe inflammatory response of the lung from either blunt force trauma, increased breathing frequency and tidal volume, or a combination of all three following contact sport may result in direct alveolar injury, pulmonary oedema and alveolar collapse (Ashbaugh *et al.*, 1967; Bernard *et al.*, 1994). The level of  $E_{drs}$  would increase; signifying potential injury to the alveoli resulting in greater stretching of the less affected.

## 1.10 MONITORING EXERCISE STRESS

Since professional sport is a business, protecting the assets or players of that business is vital for success. As a result of continual technology expansion, more time and effort is dedicated to maintaining and monitoring the health of players and athletes. Several subjective, qualitative and quantitative technologies are enlisted for both acute and chronic assessment to manage recovery around single training or competition games or events, and monitor the season-long well-being of each athlete for repeatable maximal performances.

Characterization of player health and implementation of rest and recovery provides added difficulties. Several measures are used for such a prognosis. Questionnaires about fatigue and mood (Flynn *et al.*, 1994; Urhausen *et al.*, 1998a; Urhausen *et al.*, 1998c) are noted for their sensitivity with athletes often showing an inverse “iceberg profile” in their profile of mood state (Morgan *et al.*, 1987) and self-reported “reduced capacity to act” (Urhausen *et al.*, 1998c). Biomarker analysis meanwhile, adds an extra robust level of assessment that can quantify season-long fatigue or over-training syndrome; although some studies fail to confirm changes in certain over-trained athletes (Rowbottom *et al.*, 1995; Urhausen *et al.*, 1998b). Oxidative stress, hormonal variations, enzymatic activity, immune and haematological parameters have all been proposed (Banfi *et al.*, 2006; Cunniffe *et al.*, 2011; Finaud *et al.*, 2006b; Mackinnon *et al.*, 1997). Other tools for athlete health assessment have included resting morning heart rate (Dressendorfer 1985), heart rate variability (Baumert *et al.*, 2006; Hynynen *et al.*, 2006),  $\dot{V}O_2\text{max}$  (Uusitalo *et al.*, 1998), short-term high-intensity endurance exercise (Urhausen *et al.*, 1998c), co-ordination (Kirwan *et al.*, 1988), and 1-repetition maximum strength (Fry *et al.*, 1998). These options all provide valid and research verified options for assessing the acute and chronic health of a player, however the ease of collection and assessment of these variables on a weekly basis proves difficult.

### 1.10.1 Questionnaires

Questionnaires about performance, recovery and health are subjective and qualitative. Their added advantages over other quantitative exercise stress monitoring tools are their individualistic approach. While this can be the downfall of questionnaires based on the

individual differences in perceived soreness and fatigue, it does provide an insight into the effect of exercise on a specific individual. Large inter-individual differences exist in both a clinical (Wilson *et al.*, 2001) and exercise context (Bouchard and Rankinen 2001) that can be attributed to fitness levels, familial factors, genetic profile, and to a lesser extent, age, sex and ethnicity. This variation reinforces the use of questionnaires for their interpretation by coaching and medical staff about how best to proceed with an athlete in the days and weeks following training or competition.

Questionnaires in a contact team sport where players are expected to play on a weekly basis and experience a variety of sport-related stressors such as injury, physical errors, and mental errors during training and matches (Nicholls *et al.*, 2006) are pertinent to manage acute and chronic load and stress. The added dynamic of physical impact presents a separate avenue of stress management. Questionnaires can include investigation into mood state (McNair *et al.*, 1992; Urhausen *et al.*, 1998a), perceived energy levels, muscle soreness, sleep duration, nutritional aspects, and fatigue. These types of questionnaires are predominantly based around the daily analyses of life demands for athletes (DALDA) questionnaire (Rushall 1990) which contains 26 items – nine relate to sources of stress and 17 to symptoms of stress. A professional rugby based study identified players as “worse than normal” the day after a match compared to training and rest days. This corresponded to a state of fatigue, tiredness and over-training (Nicholls *et al.*, 2009), while the stressors commonly reported are injury, mental and physical errors (Nicholls *et al.*, 2006). Similarly a questionnaire identified “playing hurt” is a universal characteristic among amateur and professional rugby union players (Liston *et al.*, 2006), while heightened stress seems to coincide with higher levels of competition (Nicholls *et al.*, 2006) which is explained by increased stress of athletes when important goals are placed under threat (Lazarus 2006).

### **1.10.2 Global Positioning System**

Global positioning system technology has been utilized in professional rugby as a means to measure and quantify workload of a player during training and match play. The information gathered is then used in conjunction with other measureable indices of workload for player management and recovery. Global positioning system is a satellite-based navigation system

that provides location and time information in all weather conditions, anywhere on or near the Earth where there is an unobstructed line of sight to four or more GPS satellites (Hofmann-Wellenhof *et al.*, 1993). Originally developed by the United States Department of Defence for overcoming limitations in previous navigation systems, GPS now provides accurate monitoring of distance covered and velocity during field and team sports (Jennings *et al.*, 2010; Johnston *et al.*, 2012; MacLeod *et al.*, 2009). Through comparison of the signal travel time of radio frequency signals sent from the orbiting satellite and the GPS receiver worn by the player, positional data is attained. The exact distance to the satellite is then calculated by multiplying the signal travel time with the speed of light. Calculating the distance to at least four satellites gives the exact position through trigonometric mathematics (Cunniffe *et al.*, 2009; Larsson 2003). The ability of GPS to quantify distance or time spent at different speeds through the Dopler shift method provides more important information pertaining to both the individual physiological characteristics of a player, and the time or distance spent at or above so-called “thresholds”. Typically the pre-determined velocity bands are company or team specific, but generally revolve around five intensities; walking, jogging, striding, high-intensity running and sprinting. The limitation of this technology is the lack of ability to relate to the individual variability amongst the athletes genetic profile or physiology. To solidify its use in stress management, a comparison with pre-calculated individual player thresholds would have to be considered in order to get a true representation of player stress.

Global positioning system units, which are no bigger than a mobile phone and sits between the shoulder blades in a specifically designed vest, includes a GPS receiver, gyroscope, magnetometer, accelerometer and heart-rate monitor. Player-load; a measurement of changes in acceleration and deceleration with impacts can be calculated using the directional changes in momentum of the individual using the following equation:

$$1) \quad \sqrt{((a_{y1} - a_{y-1})^2 + (a_{x1} - a_{x-1})^2 + (a_{z1} - a_{z-1})^2)}$$

where  $a_y$  = anteroposterior acceleration,  $a_x$  = mediolateral acceleration and  $a_z$  = vertical acceleration. Age-group, provincial, franchise and international rugby competitions have been analysed using this technology (Austin *et al.*, 2011; Coughlan *et al.*, 2011; Cunniffe *et al.*, 2009; Deutsch *et al.*, 2007; Duthie *et al.*, 2005; Hartwig *et al.*, 2011; Quarrie *et al.*, 2013;

Venter *et al.*, 2011). It not only provides quantifiable data, but pinpoints individual strengths and weaknesses alongside signs of exhaustion. These advantages have led to GPS becoming an integral part of professional rugby union team's player analysis world-wide.

### 1.10.3 Video-analysis

Video-analysis is a less precise tool for “stress” quantification in rugby union. However it is an extremely important quantitative and non-invasive piece of technology which addresses impacts in contact sport. It can be used to develop effective training regimes and enhance on-field performance. Less important in field sports such as hockey where high force impacts are not an integral part of the game, calculating the total and type of impacts during the course of a rugby game is essential for identifying players with a high workload or risk of over-training or injury. Several companies offer complex software that now pinpoint the exact moment and type of incident a player experiences. This information can be tallied up to provide an overall impact score broken down into specific sub-categories. In rugby union, these can include; tackles, tackle assists, ball carries, ball touches, and first three to a ruck on offence and defence. Professional teams then use their own weighted interpretation of the stress or work involved in each of these categories to provide an overall “workload” of a player. Combined with GPS data, this can offer an efficient and reliable source of information to assist coaching, training and medical staff on the overall “stress” experienced during a game or training session.

Video-analysis workload estimation in a rugby context has been routinely investigated (Fuller *et al.*, 2007; McIntosh *et al.*, 2010; Quarrie and Hopkins 2008). They identify rugby union as one of the most physically demanding sports in the world where total and type of impacts are position dependent. The inexpensive and stress-free nature of this technology makes it ideal for independent stress monitoring. However there is a lack of research investigating the effects of these impacts on a physiological and psychological response. The sub-categorization of impact type does not consider the position, force or angle of impact that undeniably has an effect on the physiological response. Whilst total and type of impacts can give a broad evaluation of impact stress, it does not capture all factors that may influence “workload”.

#### 1.10.4 Biomarkers

There are two types of biomarker; those used in risk prediction, and those used to screen, monitor and diagnose. The National Institute of Health (NIH) defines a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Atkinsons *et al.*, 2001). Alternatively, the World Health Organization (WHO) defines a biomarker as “almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction” (WHO 1993). The use of a biomarker can provide a quantifiable characteristic of a biological process that can be used as a clinical surrogate endpoint (Ellenberg and Hamilton 1989), or provide a snapshot of the effect of an exercise protocol (Lancaster *et al.*, 2004) or clinical treatment (Fuchs *et al.*, 1989a). They can aid in understanding the prediction, cause, diagnosis, progression, regression, or outcome of treatment of disease (Mayeux 2004), or in the context of this research, the acute and chronic stress response to rugby union.

They have several added advantages over often qualitative and subjective in nature measures currently used in professional sport. These can include and are not limited to: the provision of a full spectrum of disease, recovery and injury progression, instantaneous and initial stress analysis that reduces the degree of misclassification (Mayeux 2004), separate individual physiological variations amongst players often influence the psychophysiological response to stress. These can isolate the individual variations in perceived soreness or fatigue that coaches and medical staff heavily rely on to make judgements and facilitate recovery and re-entry into training and playing.

Development and evaluation of biomarkers in separate contexts is imperative for their validity as a screening, diagnosis and prognosis tool. Three measurements have been suggested which include the degree to which the biomarker reflects the biological phenomenon (content), the degree to which a marker measures what it claims (construct), and the extent to which it correlates with a specific disease (criterion) (Schulte and Perera 1998). Concern arises due to the large inter-individual variability among patients or athletes. Whilst



this can cause problems with diagnosis, reference ranges, and experimental response to clinical trials, it may offer a benefit for individual athlete variation in response to training or competition. The initial analysis may be broad; however continual measurement of stress to the same approximate stimuli should provide an individual reference range for a particular player or athlete.

## 1.11 INVASIVE VS. NON-INVASIVE MEASUREMENT

In medical terms, an invasive procedure is a diagnostic or therapeutic technique that requires entry of a body cavity or interruption of normal body functions. In contrast, a non-invasive technique is strictly defined when no break in the skin is created and there is no contact with the mucosa or skin break or internal body cavity beyond a natural or artificial body orifice (Mosby 2009). While both procedures offer various benefits, consistent sample collection in professional athletes in a professional setting requires an approach that is stress free, hassle free and non-disruptive. Additionally, for an analyte to be considered as a prognostic, diagnostic and acceptable marker, it has to go through five stages of evaluation:

- 1) the analytic (precision and accuracy).
- 2) diagnostic (sensitivity and specificity).
- 3) patient outcome efficacy (medical decision-making).
- 4) operational (predictive value and efficiency).
- 5) cost/benefit (societal efficacy) (Fryback and Thornbury 1991).

Availability can often cause certain restrictions when access to specific analytes is required for the assessment of exercise induced stress. Haematological parameters have been investigated extensively in rugby union (Banfi *et al.*, 2006; Cunniffe *et al.*, 2010) and provide a comprehensive overview of stress relative to acute and chronic exposure. Whilst this offers an effective representation, biomarkers often overlap providing unnecessary investigation. When quantifying changes in physiological systems such as immune function and inflammation following strenuous exercise, suppression in certain lymphocyte populations and elevated levels of inflammatory cytokines following exercise have been extensively reviewed (Pedersen and Toft 2000). Non-invasive alternatives such as urine and saliva

collection offer reliable indicators that do not restrict the ability to quantify “stress”. Urinary NP and adrenaline, sIgA, salivary cortisol and saliva testosterone for example have been shown to repeatedly provide a precise quantification and analysis of exercise induced stress and disease or illness that does not require the use of an invasive procedure (Cunniffe *et al.*, 2011; Eisenhut *et al.*, 2011; Fuchs *et al.*, 1983; Lindsay *et al.*, 2014a; Mazzeo *et al.*, 2001; Morgan 2011; Sari-Sarraf *et al.*, 2006; Walsh *et al.*, 2002). In addition, providing a measurement of the total free (un-bound) hormone concentration in the case of saliva more accurately reflects the active form in the body (Read 1989).

The stress-free nature of non-invasive procedures allows for an uncomplicated sample collection with less risk of cross-contamination. Venepuncture is quite often associated with pain and considerable distress of the athlete or patient that may affect secretion of certain stress hormones like cortisol (Okamura *et al.*, 2014; Weckesser *et al.*, 2014). This becomes especially important in studies that are quantifying stress following exercise that may lead to false-positive results and an over-estimation of the stress imposed by the exercise. Moreover, the majority of the population have been previously questioned about their preference for sample collection, all of whom would rather avoid the unpleasant connotations associated with venepuncture. Comments ranging from “extremely distressing” in children (Hands *et al.*, 2010) to “dread” in 11 out of 100 patients surveyed in a study (Keep and Jenkins 1978) indicate the general consensus toward invasive procedures in sample collection.

In a situation where a professional sporting team is the subject of a study, a non-disruptive and stress free collection of samples is required. This becomes especially important when sample collection is conducted when athletes or players have a specific ritual and are in a focussed state before a game or event. Venepuncture can become overly obtrusive and time consuming especially in a team sport where upwards of 20 players are involved. Both opposition and venue can alter the secretion of cortisol before a rugby game (Morgan 2011) which undoubtedly signifies keeping pre-game stress to a minimum. Simple urine and saliva collection procedures allow for personalised individual timing of sample collection, no unwanted increase in stress hormone concentrations, and no excessively prolonged time of collection.

There is also no requirement of a trained phlebotomist when dealing with non-invasive procedures. Unlike venepuncture that involves trained personnel having knowledge of

anatomy and physiology, non-invasive collection does not have any prerequisites. Meanwhile, the added advantage of saliva and urine over blood collection can be attributed to the simple type of collection devices. In contrast, blood collection requires the use of special sample tubes to safely collect and stabilise the blood components. These contain anti-coagulating compounds, clot activating factors, and ligand binding compounds to ensure there is no cross-contamination between tubes, whilst urine and saliva collection involves the subject emptying their bladder or salivating into a tube.

## 1.12 SALIVA

Saliva is an exocrine solution (Berkovitz *et al.*, 2002) made of 99% water (H<sub>2</sub>O) which can be considered as gland-specific or whole saliva. Differentiated by the four types of individual glands, analysis is typically completed on whole saliva secretions. It contains a wide spectrum of oral fluids, secretions from both the major and minor salivary glands, and several constituents of non-salivary origin such as gingival crevicular fluid (GCF), expectorated bronchial and nasal secretions, serum and blood derivatives from oral wounds, bacteria and bacterial products, viruses and fungi, desquamated epithelial cells, other cellular components, and food debris (Fox 1988; Kaufman and Lamster 2002; Mandel and Wotman 1975; Seward 1992; Sreebny 1988). It has protective properties, contains a variety of antimicrobial constituents and growth factors, lubricating functions, facilitates taste perception, cleanses and buffers, aids in food digestion (Mandel 1987; Shugars and Wahl 1998; Zelles *et al.*, 1995) and reflects the body's health and well-being.

### 1.12.1 Saliva Production and Composition

Saliva production per day is quantified as being within one to one and a half litres (Humphrey and Williamson 2001). It originates mainly from four pairs of glands; parotid, sublingual, submandibular and minor (Proctor and Carpenter 2007), with relative contributions ranging from four to 65 % (Chicharro *et al.*, 1998). Each gland can produce a variable amount of salts, ions and proteins (Hu *et al.*, 2004; Kalk *et al.*, 2002) that can be influenced by factors including psychological and hormonal status, physical exercise, flow rate, circadian rhythm, type and size of the gland, size and type of the stimulus, blood type, smell and taste, drugs,

age, hereditary influences, and oral hygiene (Aps and Martens 2005; Chicharro *et al.*, 1998; Schipper *et al.*, 2007; Walsh *et al.*, 2004) with composition variability dependent upon basal secretion or ANS stimulation (Kalk *et al.*, 2002). Both  $\alpha$  and  $\beta$  adrenergic receptors alongside cholinergic stimuli can modify protein concentration gland secretion through activation of the acinar cells which comprise the salivary glands (Bishop and Gleeson 2009; Carpenter *et al.*, 2000; Carpenter *et al.*, 2004). These cells can be organised into serous (secretion of a watery fluid devoid of mucins) and mucous cells (mucin-rich secretion) that secrete a fluid containing several constituents into collecting ducts.

Containing a variety of enzymes, hormones, antibodies, antimicrobial constituents, and growth factors (Rehak *et al.*, 2000; Zelles *et al.*, 1995), saliva is an ideal medium for the analysis and diagnosis of disease states and exercise stress. It is primarily composed of H<sub>2</sub>O with an abundance of weak and strong ions where their concentration is dependent on secretion stimulation type (Chiappin *et al.*, 2007). Organic non-protein compounds such as uric acid, creatinine, bilirubin, glucose, amino acids, fatty acids, amines and lactate are also detectable (Agha-Hosseini *et al.*, 2006; Chicharro *et al.*, 1998; Cooke *et al.*, 2003; Coufal *et al.*, 2003; Diab-Ladki *et al.*, 2003; Guan *et al.*, 2004; Lloyd *et al.*, 1996). Other constituents include up to 2290 proteins (Pfaffe *et al.*, 2011) comprised mainly of amylase, sIgA, carbonic anhydrase and proline-rich proteins, catecholamines (Marini and Cabassi 2002) and hormones such as cortisol and testosterone whose concentrations represent serum-free levels (Kumar *et al.*, 2005).

### 1.12.2 Transportation of Biomarkers

The transportation of saliva constituents through diffusion, filtration, or active transportation is size and charge dependent (Figure. 1.6). While most of the organic compounds like IgA and IgG are synthesised locally (Brandtzaeg 1989), blood derived compounds enter through passive diffusion of lipophilic molecules or active transport of proteins via ligand-receptor binding (Marini and Cabassi 2002).

Diffusion, the most common route for substance migration into saliva, requires biomolecules bypassing five separate barriers; the interstitial space; the basal cell membrane of the acinus cell or duct cell; the cytoplasm of the acinus or duct cell; and the luminal cell membrane (Haeckel and Hanecke 1996). Passive diffusion is both partly dependent on particle size and

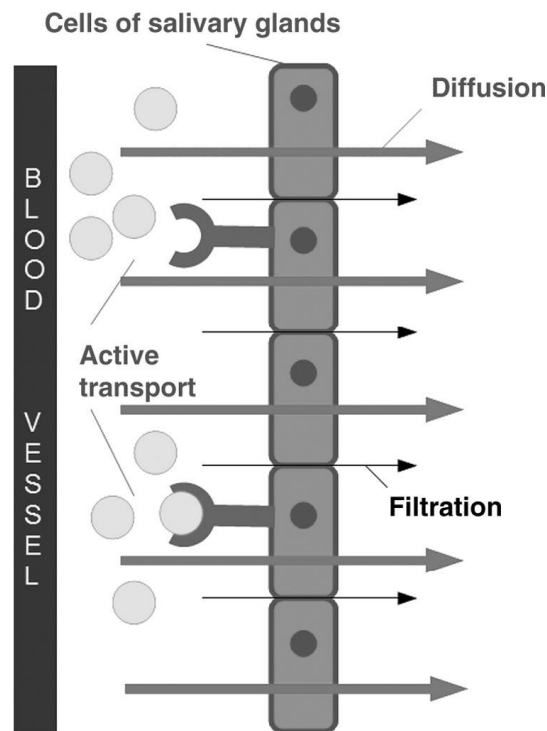
charge and not normally associated with flow rate dependence (Vining *et al.*, 1983). Therefore, those compounds that are polar or bound to carrier proteins such as albumin would struggle to enter the saliva through passive diffusion (Pfaffe *et al.*, 2011; Vining *et al.*, 1983). The porous nature of the blood capillaries allows for the migration of the biomolecules to saliva which is reliant on the permeability of the salivary glands cell membrane.

Active transport however, uses chemical energy as a mode of transport for larger or polar biomolecules to pass through the secretory cells of the glands into the saliva. Examples include IgA which is secreted by B-lymphocytes in close proximity to the salivary glands and transported across the salivary glandular epithelial cells (Lamm 1998) through IgA receptor binding. This process is increased by sympathetic activation and mobilization of the Ig receptor (Carpenter *et al.*, 2004).

Ultrafiltration is a third mechanism responsible for the transport of biomolecules into saliva that is size dependent ( $MW < 1900$  Da). For those molecules with a positive charge such as sulphated steroids, this process offers an alternative to passive transport. This diffusion can occur between acinar or ductal cells and through gap junctions between secretory units. Additionally, larger proteins such as albumin can reach the saliva through transudation from crevicular fluid or directly from the oral mucosae (Marini and Cabassi 2002).

### 1.12.3 Use in Clinical Prognosis and Exercise Stress

Saliva represents an increasingly useful auxiliary means of diagnosis due to its relative ease and stress free collection protocol, especially when blood or urine sampling is not possible. Its role and connection with several pathological and physiological states enables suitable analysis and predictions. Whilst saliva diagnostics have been predominantly utilised in the detection of oral diseases such as Sjögren (Pedersen *et al.*, 2005) and Beçhet syndrome and oral tumours (Li *et al.*, 2004), they have been proposed as reliable and accurate predictors of several other illnesses, diseases and exercise induced stress changes. The diagnosis and progression of an autoimmune disorder such as Sjögren syndrome have postulated the use of IL-6 and IL-2 as potential salivary indicators. With values significantly higher in those compromised with the disease (Streckfus *et al.*, 2001), salivary markers may provide a useful alternative.



**Figure 1.6.** The transportation of biomolecules from blood capillaries to saliva. Reproduced with permission as part of Pfaffe et al. 2011.

Usage in cardiovascular disease assessment however is not as conclusive. Identified as the underlying cause of the disease (Hansson 2005), inflammatory markers such as cTnT, CRP and amylase are quantifiable in saliva. Their sensitivity and relativity is questionable however; though amylase has been demonstrated to be a more precise and simple end-point of heart rate change in patients in stressful conditions (Chatterton *et al.*, 1996). Furthermore, salivary biomarkers have also been postulated as non-invasive alternatives in endocrinology (van Honk *et al.*, 1999), infectious diseases (Martínez *et al.*, 1999), nephrology (Lloyd *et al.*, 1996), oncology (Tavassoli *et al.*, 1998), drug monitoring (Slavkin 1998), cancer (Streckfus *et al.*, 2000) and psychiatry (Yamada *et al.*, 1998).

Exercise induced stress provides a different, yet similar avenue for the use of salivary biomarkers in prediction and evaluation. Their use can provide both an acute assessment of physiological and psychological stress to a game or event (Allgrove *et al.*, 2008), or chronic assessment of over-training during a season or competition (Cunniffe *et al.*, 2011). Salivary markers have identified fatigued and over-trained swimmers during the course of a competitive season (Gleeson *et al.*, 1995), able to identify those at risk of a URTI in elite rowers, yachtsmen and soccer players (Han *et al.*, 2010; Mortatti *et al.*, 2012; Neville *et al.*, 2008; Putlur *et al.*, 2004), marathon runners who become immunocompromised following a race (Nieman *et al.*, 2002), elite rugby and rugby league players suffering from over-reaching, over-training or post-match fatigue (Elloumi *et al.*, 2003; Maso *et al.*, 2004), level of psychophysiological and catabolic stress associated with exercise of a high intensity or intermittent nature (Beaven *et al.*, 2008; Ghigiarelli *et al.*, 2013; Kraemer *et al.*, 2001b), monitor changes in the circadian rhythm of trained athletes following strenuous exercise of different natures (Hayes *et al.*, 2010), and monitor rugby based performance (Cook *et al.*, 2014). Their measurement has a clear use in sports and exercise performance analysis that can be performed on a regular non-invasive basis.

#### 1.12.4 Limitations

As a diagnostic medium, saliva has certain limitations; specifically in the medical field where diagnostics rely heavily on combinations of biomarker panels used as screening tools to improve overall reliability. Its relative ease of collection, stress free procedure and development of modern precise technologies however has expanded its usefulness in disease prognosis with proof-of-principle studies conducted on patients with HIV and Hepatitis (Chaita *et al.*, 1995; Emmons 1997; Malamud 1997).

As a consequence of the diurnal and circadian variations of certain biomolecules present in saliva (Johnston *et al.*, 1980), concentrations do not always reliably reflect the concentrations of these molecules in serum. Therefore it is imperative when analysing exercise stress to plan a precise collection protocol before the experiment to understand the individual biological variation of a subject, or take multiple samples at the same time to provide meaningful results (Read 1989).

Biomolecules in saliva that rely on active transport are generally flow rate dependent meaning exercise-induced changes have to be corrected for saliva flow rate. Studies have identified differences in post-exercise changes in sIgA in cyclists completing an exercise protocol in a cold environment. Saliva flow decreased significantly following exercise resulting in an increase in sIgA concentration, however when corrected for flow rate, the protocol seemingly caused immune suppression calculated by the secretion rate of IgA (Walsh *et al.*, 2002).

Salivary composition can also be influenced by the method of collection and the degree of stimulation of salivary flow (Hofman 2001; Kaufman and Lamster 2002). Careful consideration has to be given to the methodology of sample collection due to the risk of over-stimulation providing a false-positive or negative result (analyte dependent) using cotton absorbent materials, hydrocellulose or acidic based stimulation (citric acid) (Gallagher *et al.*, 2006; Kirschbaum *et al.*, 1992; Shirtcliff *et al.*, 2001; Strazdins *et al.*, 2005). However, some devices such as Sarstedt-Salivette<sup>®</sup> have been shown to reflect total and free steroid concentrations more accurately than the traditionally accepted passive-drool technique (Poll *et al.*, 2007). Furthermore collection time duration and position of collection can significantly affect concentration and secretion rate of specific analytes (Beltzer *et al.*, 2010).

Saliva contains analytes in concentrations that are several-fold lower than blood (Chiappin *et al.*, 2007; Christodoulides *et al.*, 2005; Marini and Cabassi 2002; Miller 1993) which reduces the risk of danger or infection when dealing with potentially hazardous antigens related to HIV or Hepatitis (Major *et al.*, 1991). It is for this reason advanced technologies are required to quantify any changes in analyte concentration. The development of competitive ELISA's today can detect concentrations of salivary steroids as low as two pg/mL with a sensitivity limit of 1 pg/mL, while liquid-liquid extraction by either diethylether ((C<sub>2</sub>H<sub>5</sub>)<sub>2</sub>O) or dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>) provides an added detection step extracting polar substances from the saliva (Granger *et al.*, 2007). This ability allows the detection of any small changes following exercise stress that may represent a significant change. As a result of the large concentration differences between blood and saliva, contamination through use of mouth guards or impact related gum trauma causing bleeding can significantly alter the concentrations of specific analytes (Hofman 2001; Kivlighan *et al.*, 2005). This highlights the



need for robust saliva collection protocols as they are imperative for precise and accurate interpretation of results.

### 1.12.5 Saliva Correction

Typically, saliva compounds are expressed in four different forms;

1) Absolute concentration ( $\mu\text{g/mL}$ ).

2) Secretion rate ( $\mu\text{g/min}$ ) to account for those biomolecules (IgA, DHEAS) (Hofman 2001) affected by flow rate. Some evidence suggests that exercise induced changes in flow rate are psychological and related to parasympathetic withdrawal rather than sympathetic activation (Bishop *et al.*, 2006; Bosch *et al.*, 2002). Exercise is established as causing a decrease in saliva volume (Bishop *et al.*, 2000) that may cause a concentration effect of a marker. This correction has been presented following a 160 kilometre run where IgA concentration had not changed, but a 50 % decrease in secretion rate was observed (Nieman *et al.*, 2003a).

3) Concentration relative to total protein content ( $\mu\text{g.mg.protein}$ ) to account for oscillations in protein content relative to changes in biomolecule concentration. The assumption is that total protein content does not change in response to exercise. A decrease in sIgA was observed following cross country skiers and cyclists which further decreased when corrected for total protein (Mackinnon *et al.*, 1989; Tomasi *et al.*, 1982). However it has been suggested that correction with total protein is inappropriate and misleading (Blannin *et al.*, 1998). Studies have identified no change in sIgA or flow rate following intense exercise, but an increase in total salivary protein which indicates an evaporative loss of saliva when breathing through the mouth during exercise (Moreira *et al.*, 2009a; Walsh 1999).

4) Concentration relative to saliva osmolality ( $\text{mg.mOsm}$ ) to account for low salivary flow rates (Sari-Sarraf *et al.*, 2007). Indicating exercise as detrimental to saliva flow quantity but not quality, and with the total protein content in saliva less than one percent (Blannin *et al.*, 1998), expressing a salivary biomarker as a ratio to osmolality may be an appropriate means of expression.

The presentation and reference range determination of salivary biomarkers is an extremely important aspect of exercise induced stress analysis. This may explain some of the variation

involved when comparing and contrasting results within and between sports. While most exercise studies will suggest the intensity of the protocol or game is the determining factor in changes in salivary biomarkers of physiological and psychological stress (Nehlsen-Cannarella *et al.*, 2000; Reid *et al.*, 2001; Sari-Sarraf *et al.*, 2006), it is possible the difference may be attributed to either the collection protocols in place and mishandling of samples, or the expression of the marker in the processes described.

## 1.13 URINE

Urine is an aqueous solution of an organism that is used to excrete the by-products of cellular metabolism through kidney filtration whose appearance is based on colour, foaming, odour and clarity (Cook *et al.*, 2000).

### 1.13.1 Production and Transportation

Urine production is controlled by the kidneys in a series of events; filtration, reabsorption, secretion and excretion (Figure. 1.7). The nephron is the functional unit of the kidney responsible for urine production whose dominant vascular component is the glomerulus; a tuft of capillaries that filters protein-free plasma from an afferent arteriole into the tubular component composed of the Bowman's capsule, proximal tubule, loop of Henle, distal tubule and collecting duct. This is the first step in urine formation where approximately 20 % of the plasma that enters the glomerulus is filtered into Bowman's Capsule at a rate of 125 mL/min. Chronic kidney disease is considered when this value is equal or less than 63 mL/min (Clase 2006). Those substances that are important to the body are reabsorbed in the second step of urine production where on average 178.5 of the 180 litres of plasma filtered each day is selectively reabsorbed from the tubular lumen into the peritubular capillary plasma. The last step in the process of urine production is tubular secretion, a process whereby substances are actively transported from the peritubular capillaries that carry the remaining unfiltered plasma (80 %). The final component is a concentrated solution of non-selectively chosen constituents that is passed into the renal pelvis for excretion.

Glomerular filtration, or the first step, is a size and charge selective membrane that retains blood cells and plasma proteins that cannot filter through the capillary pores and are essential

in monitoring kidney function. Acting like a molecular sieve, plasma is filtered through a series of three membranes driven primarily by the capillary blood pressure; capillary endothelium, basement membrane and filtration slit into Bowman's Capsule where debate still continues over the primary limiting factor. As a result of the size exclusivity, proteins larger than 10 kDa struggle to fit through, while anything over 100 kDa will never pass which is a critical factor in the early diagnosis of kidney damage.

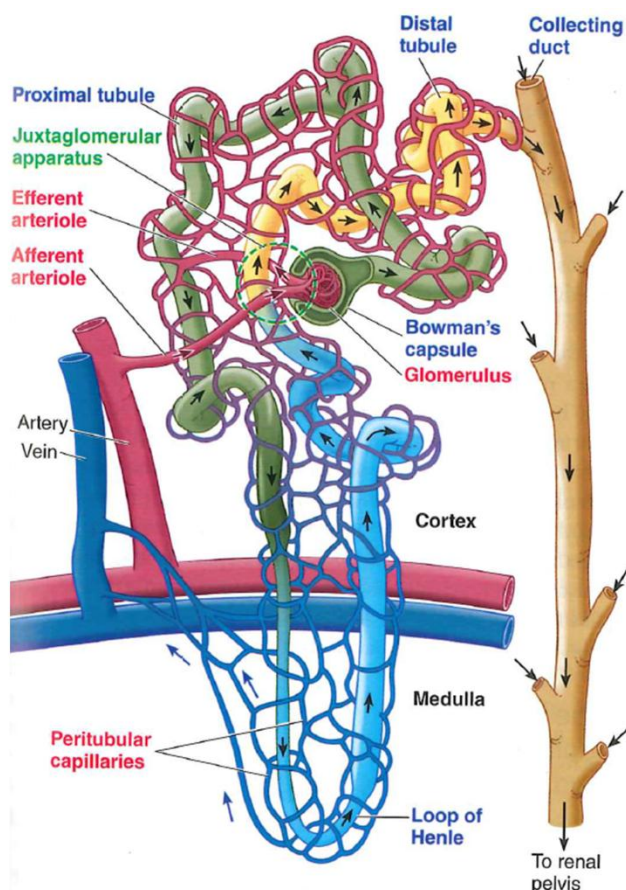
Tubular reabsorption is similar in its selectivity of substances with reabsorption rates of H<sub>2</sub>O at 99 %, at 100 % for sugar and 99.5 % for salts. Tubules have a high capacity for reabsorbing substances that are needed for the body or to maintain the proper composition and volume of the internal fluid environment (Sherwood 2008). Transport across the one cell thick tubule occurs through both passive and active transport where five separate barriers must be crossed; the luminal cell membrane, cytosol, basolateral cell membrane, interstitial fluid and the capillary wall. Passive transport requires no chemical energy for substance movement due to the net movement down electrochemical and osmotic gradients. Active transport however, utilizes specific pumps to move needed substances against an electrochemical gradient that include glucose, amino acids and essential electrolytes.

Tubular secretion, or the third and final step, is the last chance for a substance to be secreted from the peritubular capillaries into the tubules through active and passive transport. Important for maintaining ion balances (H<sup>+</sup> and K<sup>+</sup> ions) and expelling foreign compounds, it is an important and highly regulated mechanism for hastening elimination of unwanted compounds. An example includes the organic ion secretory systems that regulate the secretion of many drugs. In order to keep up with the excretion rates, a repeated frequency is required to maintain plasma levels.

### 1.13.2 Urinary Biomarkers

As an aqueous solution, urine is predominantly H<sub>2</sub>O ( > 95 %), electrolytes, metabolic excretory products and other organic and non-organic compounds (Cook *et al.*, 2000). While these include nitrogen containing compounds, elements such as phosphorous, potassium, sodium, sulphur, calcium, cobalt, mercury, chlorine, magnesium, iron, boron, aluminium, cadmium, copper, nickel, lead and zinc (Kirchmann and Pettersson 1994), it also has

compounds capable of providing useful information regarding certain illnesses and psychophysiological changes following exercise induced stress.



**Figure 1.7.** Components of a mammalian nephron. Reproduced with permission as part of Human Physiology: from cells to systems (Sherwood 2008).

Glomerular filtration selectivity is a defining characteristic and key determinant in disease pathology investigating kidney damage. Kidney damage is typically assessed through urinary albumin concentration where values ranging from 30 to 300 mg/serum.albumin/day may be an early sign in patients with diabetes mellitus, hypertension or an indication of cardiovascular disease (Forman *et al.*, 2012; Group 2000; Hillege *et al.*, 2002). Its molecular weight (66.5 kDa) prevents its filtration in a healthy kidney making it an ideal measurement

of kidney function that is occasionally seen following strenuous exercise (Clarkson 2007). It is not only a sign of kidney disease but can also result in tubular and interstitial damage contributing to the progression of chronic renal disease (Thomas and Schreiner 1993). Similarly, haemoglobin, an iron containing protein responsible for oxygen (O<sub>2</sub>) transport and similar in size (68 kDa), can represent haematuria or hemoglobinuria. This is often seen in disease (Fairley and Birch 1982) or athletes following intense exercise (Groop *et al.*, 1990; Mercieri *et al.*, 2011; Siegel *et al.*, 1979) signifying renal injury. It is however duration and intensity dependent (Sarhadi *et al.*, 2011) with the resolution characteristically rapid and self-limiting (Siegel *et al.*, 1979).

Myoglobin, a similar O<sub>2</sub> transportation heme protein, is smaller in size (17 kDa) and filtered through the glomerulus without any hindrance providing its plasma binding capacity has been exceeded (Vanholder *et al.*, 2000). Once the proximal tubules reabsorption rate has been exceeded, myoglobin appears in the urine turning it reddish-brown in colour. As a protein specific to muscle, its detection in urine provides a good quantification of trauma induced muscle damage commonly seen following strenuous exercise (Uberoi *et al.*, 1991), myocardial infarction (Donald *et al.*, 1977) and drug toxicity (Bourgeois and Richards 2013). This not only provides an indication of muscle damage severity but can also directly cause obstruction and renal dysfunction (Zager 1996b).

Steroidal compounds including natural and synthetic varieties are filtered through the glomerulus without any limitation. Identification of these compounds within the urine can provide useful information regarding diseases such as hypercortisolism (Cushing's Syndrome), psychiatric disorders (Kiecolt-Glaser *et al.*, 1984; Virkkunen 1985) and post-traumatic stress disorder (PTSD) (Yehuda *et al.*, 1993), alongside the primary identification system used by the World Anti-Doping Agency (WADA). The testosterone:epitestosterone ratio is often used in the detection of doping athletes (Saudan *et al.*, 2006) with concentrations exceeding one to four considered above normal.

Other urinary biomarkers in the quantification of exercise induced stress can include isoprostanes in the measurement of oxidative stress (Margonis *et al.*, 2007), IL-6 in the measurement of an inflammatory response (Suzuki *et al.*, 2002), and adrenaline in the investigation of the ANS (Christensen *et al.*, 1976). The filtration process and non-invasive

nature of collection involved in urine sampling makes it an ideal matrix for the detection of exercise-induced stress.

### 1.13.3 Volume Correction

While measurement of salivary biomarkers requires correction for flow rate, urine requires volume correction. Hydration levels are known to effect the concentrations of urinary biomarkers. Hypo-hydration has a concentration effect while hyper-hydration has a dilution effect. In exercise specifically, the consumption of H<sub>2</sub>O and carbohydrate-electrolyte formulations are known to influence temperature regulation, physiological strain and endurance during exercise (Ladell 1955; Montain and Coyle 1992; Pitts *et al.*, 1944; Sawka *et al.*, 1984). This is a key indication of how vital hydration status is for exercise performance and therefore the challenges associated with urinary biomarker correction and hydration status.

It is critical when presenting the concentration of a selected marker in relation to a reference range to correct for the hydration level of a subject, patient or athlete. This is especially important when dealing with a severe illness or analysing the urine of suspected doping athletes in a controlled competition. The two most commonly utilized methods for urine volume correction involve creatinine and specific gravity (SG).

#### 1.13.3.1 Creatinine

Creatinine is a spontaneous breakdown product of creatine and creatine phosphate (Allen 2012) during muscle cell metabolism present in serum, erythrocytes, cerebrospinal fluid and all bodily secretions (Myers and Fine 1919; Schumann 1931). Originally identified in 1847 (Liebig) and produced initially through heating of creatine in 1885 (Horbaczewski), creatinine is now recognised and utilised as a principal compound for urine volume correction and hydration status. It is also a diagnostic marker of muscular dystrophy (Levene and Kristeller 1909) and impaired renal function (Crowe and Hatch 1977) providing a standardised collection protocol is used.

Creatinine clearance was initially thought to be achieved through glomerular filtration that was neither reabsorbed or augmented by secretion (Rehberg 1929). Proximal tubular secretion however is known to be partly involved in its clearance (Bauer *et al.*, 1982). Its constant secretion rate (Shaffer 1908) and easy quantification are two of its key properties that allows it to be used as an indicator of hydration status (Carrieri *et al.*, 2000; Cone *et al.*, 2009), while lean body mass (Clark *et al.*, 1951; Forbes and Bruining 1976), age (Rowe *et al.*, 1976) and renal function are contributing factors in its clearance that have to be considered.

The quantification of urinary creatinine was first established in 1886 (Jaffé) where the formation of a red colour was observed when creatinine reacted with picric acid in an alkaline medium. Over the past century, the reaction has been subjected to numerous experimental studies including chromatographic and spectral understanding of the reaction (Blass and Thibert 1974; Buncel *et al.*, 1968) that has identified factors such as temperature, pH (Owen *et al.*, 1954), and alkali (hydroxide) concentration (Tillson and Schuchardt 1953) as influential. Today the reaction is utilised heavily with specific modern technological advancements (Randviir *et al.*, 2013). Whilst this reaction is heavily used, other quantification methods include the reaction with o-nitrobenzaldehyde (Van Pilsum *et al.*, 1956), reverse phase HPLC (de Castro *et al.*, 2004; Tsikas *et al.*, 2004; Wachter *et al.*, 1979), cation-pairing HPLC (Paroni *et al.*, 1990), mass-spectrometry and gas-chromatography (Tsikas *et al.*, 2010). The advantage of chromatography is the simultaneous determination of creatinine with the analyte that requires creatinine correction. Usually analysed at 234 nm by ultra-violet detection, the method has been used effectively in the quantification correction of NP (Fuchs *et al.*, 1982), desmosine (Ma *et al.*, 2003), catecholamines and metanephrines (Abeling *et al.*, 1984).

There are a number of limitations associated with urinary creatinine correction. Evidence has identified higher levels of urinary creatinine in the urine of men or lean individuals than women and obese individuals respectively (Baxmann *et al.*, 2008; Clark *et al.*, 1951; Garn and Clark 1955). This indicates lean body mass as a major factor in its excretion. Furthermore, those of African-American descent excrete five percent more creatinine per weight than those of European descent, older individuals and those with renal impairment excrete less urinary creatinine than the young and healthy (James *et al.*, 1988). Individuals

who are subjected to a creatine-free or excess creatine diet show a gradual decline and increased excretion respectively (Calloway and Margen 1971; Crim *et al.*, 1975). The combination of all these variables identifies the need to ascertain the normal excretion of creatinine for each individual, even though the co-efficient of variation (CV) for individual creatinine excretion can vary from four to 50 % (Bailey and De Wardener 1970; Edwards *et al.*, 1969; Ram and Reddy 1970).

Exercise provides another layer of complexity for creatinine volume correction. Urinary creatinine has been demonstrated to increase by as much as 50-100% following a strenuous six mile run, 100 km marathon and 70 – 90 km cross country ski race (Anderson *et al.*, 1982; Decombaz *et al.*, 1979; Refsum and Strømme 1974). It is difficult to ascertain whether observed changes in a selected analyte are negatively affected by this change. A study (Paul *et al.*, 1989) investigating the excretion of the muscle damage marker 3-methyl histidine following resistance exercises showed a decrease in concentration in conjunction with an increase in serum myoglobin and CK. It is unusual that a change in muscle tissue damage occurs without skeletal muscle protein degradation. This may be a result of the likely increased urinary creatinine concentration that has been shown to increase following resistance training; which presents the potential difficulties associated with urine volume correction by creatinine. In rugby union, the large aerobic and anaerobic component of the game may stimulate large increases in creatinine that could theoretically affect the interpretation of any meaningful urinary biomarker. However WADA has adapted the use of SG in their quantification of urinary steroids and doping analysis (World Anti-Doping Agency 2004).

#### 1.13.3.2 Specific Gravity

Specific gravity is the ratio of the density of a substance to the density of a reference substance measured optimally through refractometry (Osborne and Stevens 1999). Urine specifically can be compared to distilled H<sub>2</sub>O as a reference and is known to increase in SG with solute concentration (Osborne and Stevens 1999). This varies with the total mass of solutes that depends not only on the number of particles present, but also on their molecular weight (Miller *et al.*, 2004). The advantages of urinary SG include its cost and time efficient



measurement using a simple hand-held refractometer in comparison to the time consuming and costly assays associated with creatinine.

Since 1908, creatinine has been used as the method for correcting analyte concentrations in urine due to its “constant rate” (Shaffer 1908). As a result of the known variations in creatinine excretion, SG has been sought after as a viable alternative. The minimal studies that have compared creatinine and SG have shown SG performs as well as creatinine in correcting urinary analyte concentrations (Berlin *et al.*, 1985; Carrieri *et al.*, 2000; Miller *et al.*, 2004; Suwazono *et al.*, 2005) and assessing patient and pre-exercise hydration status (Osterberg *et al.*, 2009; Silva *et al.*, 2010; Simerville *et al.*, 2005). Its limited use is a result of finite research that has not elucidated reference ranges so critical for diagnosis. Quantifying urinary proteins with SG correction can of course provide information about kidney disease, hypertension, cancers and diabetes (Newman *et al.*, 2000).

In the field of sports testing, the Levine-Fahy equation (2) is used to correct fluctuating urinary concentrations and adjust them to a reference value for a specific population (Levine and Fahy 1945).

$$2) \text{Concentration}_{\text{SG normalized}} = \text{Concentration}_{\text{specimen}} \cdot (\text{SG}_{\text{reference}} - 1) / (\text{SG}_{\text{specimen}} - 1)$$

The WADA uses a SG of 1.020 (Goldberger *et al.*, 1995) for normalisation of testosterone precursors, metabolites, and other endogenous steroids in urine (World Anti-Doping Agency 2004). Other reference values ranging from 1.018 to 1.024 have also been used (Araki *et al.*, 1990; Barber and Wallis 1986; Levine and Fahy 1945; Miller *et al.*, 2004) that are population dependent, while values < 1.0010 or > 1.020 indicate relative hydration and dehydration respectively (Kavouras 2002). These guidelines do possess complications. A study measuring SG before two separate marathons in a large cohort identified 46 % of the runners would be considered dehydrated which seems unlikely considering the magnitude of the impending exercise (Stover *et al.*, 2006).

Similar limitations are associated with urinary SG quantification. As a correction method, it may not be appropriate for individuals with diabetes mellitus and nephrotic syndrome. These cause high concentrations of heavy molecules known to affect SG and the potential

underestimation of a urinary analyte (Osborne and Stevens 1999; Parikh *et al.*, 2002; Voinescu *et al.*, 2002). The effect of exercise on SG however is not well understood, although it can be assumed the aerobic, anaerobic and physical impacts of rugby union and other contact sports would significantly affect the density of urine. Furthermore, the contraction and expansion of urinary constituents due to temperature and pressure requires the measurement of SG to be conducted in a uniform manner (Alessio *et al.*, 1985), while values  $< 1.002/1.003$  or  $> 1.003$  are considered to “dilute” or “concentrate” for accurate correction (Alessio *et al.*, 1985; Cone *et al.*, 2009; Elkins *et al.*, 1974; Sauer and Paulson 1991).

## 1.14 BACKGROUND TO RESEARCH

Research development was influenced by several factors that determined its direction and evolution for future use in a sporting and clinical context. The development of the research team was established to provide expertise in several key fields; intensive care, mechanical engineering, steroid and immunobiochemistry, exercise physiology, and medical biochemistry. With a vast array of expertise, we were able to cultivate a clear, pre-determined direction of the proposed research that successfully utilised each of these fields to their fullest capacity.

After the formulation of the research plan and team, Associate Professor Nicholas Gill (All Blacks strength and conditioning coach) was approached because of his current position and knowledge of impact related sport. He provided us with advice concerning exercise biochemistry research and its potential applications. It was evidently clear there was a vast majority of research investigating the effects of exercise changes in both physiological and psychological stress. Lacking from the research however, was the practical applications from this research and how it could be used to improve the performance of an athlete. We were also aware of a predictive model that was being developed by mechanical engineers and intensive care physicians at the University of Canterbury and Christchurch Hospital to manage patients on an individual basis based on illness-induced lung inflammation. The idea of a predictive model for the acute and chronic stress assessment following exercise was

established coincidentally, whilst also providing us some interesting research regarding a novel approach to testing exercise stress.

Nicholas Gill further mentioned the requirement for a non-invasive approach. While we were prepared to complete venepuncture sampling and plasma analysis, it was imperative no blood was to be taken from professional rugby players. It was decided that urine and saliva were the only possible options for assessing the psychophysiological state of a player. This dramatically altered our thought process, and caused us to reconceptualise and sizeably reduce the amount and type of biomarkers that could provide the team with a comprehensive quantitative assessment.

Research of this nature is undoubtedly expensive. We therefore had to restrict and meticulously plan the research in order to accommodate. There was an added incentive for producing the most economical sampling protocols that still allowed us to provide a complete overview. Careful selection of the most informative, unique, non-overlapping and economical biomarkers available was discussed and decided upon. They provided a complete psychophysiological assessment of an athlete, in order to develop efficient, repeatable and reliable assays.

A private donation and a scholarship provided by St George's Hospital and Heart Centre (2003) Ltd. allowed us to develop a proposal for the Canterbury Rugby Union and Canterbury Crusaders franchise. The donation permitted the development of a research plan that spanned the end of the 2013 ITM Cup and 2014 Super 15 campaign. It delivered significant data from several games for the acute and chronic player response as well as predictive ability of the data of an individual player's health.

## **1.15 INTENDED PROCESS OF INVESTIGATION**

There is substantial information pertaining to the acute and chronic physiological and psychological response to exercise stress. This is applicable to the majority of sports, as well as specifically designed exercise studies investigating various conditions. While this presents valuable information about the response of the individual and group, the application of the data for athlete stress and health management and the cross-over effect into monitoring post-operative patients or trauma victims has not been considered. Therefore this thesis will be

divided into eleven separate segments that provide a story of the effect of intense exercise on both the acute and chronic response, and the potential applications for the use of psychophysiological biochemical analysis in professional rugby union and elite athlete management.

After independent consultation, it is evidently clear a non-invasive and stress free approach is a necessity for monitoring professional rugby players; a surrogate for the development and research of biochemical marker specificity and reliability. After careful selection, a set of markers was identified as unique and non-overlapping; sIgA, cortisol, myoglobin and NP. Salivary immunoglobulin A, cortisol and myoglobin are all assessable through credible ELISA's. Neopterin is measured in the Free Radical Biochemistry Laboratory at the University of Canterbury for research attempting to disseminate the formation of atherosclerotic plaques. Therefore a more robust approach is required for urine detection. As part of **STUDY ONE**, we intend to:

- 1) Develop an alternative HPLC technique using strong-cation exchange (SCX) based chromatography for NP detection.
- 2) Evaluate the effectiveness of urine volume correction techniques (creatinine and specific gravity SG) following exercise
- 3) Utilise these techniques to provide information regarding the potential elevated concentrations of NP and 7,8-dihydroneopterin over the course of a week of high intensity resistance training in competitive, natural (no use of banned substances) body building.

Subsequent to the development of an alternative HPLC method for NP detection, we aim to ascertain the sensitivity, level and time course changes of the selected biomarkers in a non-invasive and stress free manner following an elite amateur rugby game as part of **STUDY TWO**. By understanding the time course changes of the selected biomarkers at the amateur level of competition, a precise and cost-effective method can be established for a professional team to maximise sample collection and analysis interpretation.

This level of competition also allows for an expansion of “stress” analysis and the opportunity to develop alternative tests. In the hope of expanding into elite sport, we intend to analyse lung inflammation as a possible substitute or addition to monitor exercise stress

using a commercially available ventilator as part of **STUDY TWO**. Typically used in the critically ill to provide breathing support and understand the inter-individual variation among patients in lung capacity and disease severity and progression, its use in conscious athletes as a means of measuring stress is investigated.

Following the establishment of the time course changes at the amateur level, we intend to expand into the professional game as part of **STUDY THREE** to measure and report both the acute and cumulative psychophysiological stress response of each individual player and group. Its purpose is to observe whether the sampling procedure and use of these markers in a professional setting is possible for the prediction of stress and management of player recovery in a non-invasive manner.

Succeeding the initial professional study, we intend to expand the muscle damage and oxidative stress relationship discovered at the franchise level of New Zealand rugby as part of **STUDY FOUR**. Currently hypochlorite is the only known compound capable of oxidizing 7,8-dihydroneopterin to NP; although other possible mechanisms are suggested but unexplained to date. Using four games throughout the season and some experimental research, we hope to uncover potential alternative mechanisms of NP production through impact induced myoglobin release and the simultaneous change in urinary pterins.

Global positioning system technology is used as a tool by professional teams to manage workload and identify weaknesses and signs of exhaustion. As a guide to aerobic and anaerobic work by a player, we intend to identify as part of **STUDY FOUR** whether there is a relationship between total distance, distance covered at pre-determined velocity bands and player-load during two consecutive games with changes in the specific cardiovascular stress marker NT-proBNP. This study aims to provide original data explaining cardiovascular stress and whether GPS technology is a reliable indicator or predictor of this stress.

We also propose to expand on the selected markers cultivated throughout this thesis to monitor the seasonal changes in psychophysiological stress throughout an entire season of professional rugby as part of **STUDY FOUR**. Over-training and reaching is normally assessed through performance measures and questionnaires, so we hope to provide a quantitative alternative through changes in cortisol, sIgA and NP and determine whether they are capable of recognising signs of over-training or reaching.

We similarly intend to expand on the positional demands of professional rugby as part of **STUDY FOUR**. Extensive literature is available on the contemporary demands of the game; however minimal data is available between positional demands per minute of play, paralleled with how this corresponds to changes in psychophysiological stress. Therefore we want to ascertain whether GPS and video-analysis data corresponds to changes in the selected biomarkers of stress between positions.

Recovery intervention research is one of the most extensively covered exercise physiology aspects and is pertinent to managing the performance and recovery of elite athletes. We therefore intend as part of **STUDY FOUR** to utilise the extensively covered biomarkers throughout this thesis to identify the effectiveness of varied recovery strategies following several professional rugby union games. These include cold water immersion (CWI), compression, sleep, stretching, active recovery and nutrition.

**STUDY FIVE** expands on the rugby related research. It became evidently clear a method is required for myoglobin quantification in a clinically relevant range. ELISA technology has several limitations ranging from detection limits to dilution influences. We therefore intend to develop a new, cost-effective, simple and reliable method using HPLC, and examine its reliability during a mixed martial arts competition. Mixed martial arts should be a worthy option for testing this new methodology due to its intense physicality. Neopterin and total NP will also be analysed to gauge oxidative stress and inflammation respectively. We will also aim to establish the effectiveness of CWI as a simple means of reducing recovery time following trauma induced exercise and identify whether the hypothesized suppression in total NP production is a result of reduced peripheral blood flow and vasoconstriction; or a combination of suppressed macrophage activation.

The measurement of several key physiological and psychological markers has the ability to provide a quantitative stress assessment of an athlete that allows for more precise monitoring, management and recovery. The measurement in urine and saliva should provide both an acute and chronic level of assessment that is lacking in professional sports where there is such a great emphasis on developing an athlete for a specific purpose. Too often subjective and qualitative measures are used to assess workload and perceived effort; however the non-invasive, stress-free and non-disruptive approach of these key biochemical markers and methods could provide an alternative. The high force and frequency impact of rugby union in

conjunction with its intermittent nature identifies it as one of the most physically demanding field sports in the world. This study should provide an extremely robust model for the testing of the selected markers of this research that could then potentially be utilised in a clinical setting for the assessment and quantification of stress in post-operative patients and trauma victims.

# Chapter 2

## Materials and Methods

### 2.1 MATERIALS

#### 2.1.1 Chemicals

All solutions were prepared in de-ionised and ultra-filtrated H<sub>2</sub>O from a NANO-pure ultra-filtration system supplied by Barnstead/Thermolyne, IA, USA or Milli-Q<sup>®</sup>, Millipore, MA, USA. All reagents used are of analytical grade or better unless stated otherwise.

Acetic Acid (glacial)	JT Baker, Mallinckrodt Baker Inc
Acetonitrile	BDH Chemicals Ltd
Ammonium phosphate bi-basic minimum 98%	Sigma Chemical Co.
Biopterin	Schircks Laboratory
Cortisol	Sigma Chemical Co.
Dichloromethane	BDH Chemicals Ltd.
7,8-dihydroneopterin	Schircks Laboratory
7,8-dihydroxanthopterin	Schircks Laboratory
Ethylenediaminetetraacetic acid (EDTA)	BDH Chemicals Ltd
Ferrous Sulfate	Merck
Gelatin	Merck
Hydrochloric acid	Merck
Iodine	BDH Chemicals Ltd
L-Ascorbic acid	Sigma Chemical Co.
Methanol	Merck
Myoglobin (human)	Abcam (ab96036)
Neopterin	Schircks Laboratory
Orthophosphoric acid (85%)	BDH Chemicals Ltd
Sodium Chloride	Merck
Sodium dihydrogen phosphate monohydrate	Scharlau Chemie S.A.
Sodium hydroxide	Merck
Sodium phosphate dibasic	Sigma Chemical Co.



Sulfuric acid	Merck
3,3',5,5'-Tetramethylbenzidine (TMB)	Sigma Chemical Co.
Thiomersal	Sigma Chemical Co.
Tween 20	Sigma Chemical Co.
Xanthopterin	Schircks Laboratory

### 2.1.2 Antibodies

Mouse monoclonal antibody to myoglobin	Abcam (ab19607)
Rabbit polyclonal antibody	Abcam (ab78841)
Goat anti-rabbit IgG-HRP	Jackson ImmunoResearch Laboratories
Cortisol monoclonal antibodies	(Lewis <i>et al.</i> , 1992)
Rat anti-mouse IgG1-peroxidase	Jackson ImmunoResearch Laboratories

### 2.1.3 ELISA Kits

Secretory IgA	Salimetrics
NT-proBNP	Mybiosource

### 2.1.4 Analytical Equipment

#### 2.1.4.1 HPLC Systems

High performance liquid chromatography (HPLC) is a high pressure analytical technique that separates, identifies and quantifies components of a sample mixture through adsorption. This is accomplished through pumping of a pressurized liquid solvent through a column with a solid adsorbent material that partitions analytes based on their different degrees of interaction with the adsorbent material. Solvent composition, oven temperature and adsorbent material composition all play a significant role in the separation process through alterations in the interaction effect. High performance liquid chromatography is used in several fields including medical, legal, research and manufacturing purposes (Gerber *et al.*, 2004) with

multiple types of chromatography available. The samples collected were subjected to HPLC analysis using two separate systems from Shimadzu<sup>TM</sup> Corporation, Japan.

Shimadzu SCL 10A (system 1): fitted with the controller SCL-10A VP, a solvent delivery module LC-10AD, a fluorescence detector RF-10AXL (xenon lamp), UV-Vis detector SPD-10A (deuterium lamp), autosampler SIL-10A with a column oven CTO-10A and DGU-14A on-line degasser.

Shimadzu CBM 20A (system 2): fitted with a central bus module CBM-20A, a solvent delivery module LC-20AD, a fluorescence detector RF-10AXL (xenon lamp), UV-Vis detector SPD-M20A (deuterium lamp), autosampler SIL-20ACHT with a column oven CTO-20A and a degasser DG-20As.

All urine samples collected were analysed in duplicate, while experimental treatments were analysed in triplicate unless mentioned otherwise. Peak areas were determined using LC solutions<sup>TM</sup> software.

#### 2.1.4.2 ELISA System

Enzyme-linked immunosorbent assay is an analytical technique that utilizes antibodies and colour change to selectively quantify a substance in a sample. The antigen or analyte of importance is either adsorbed non-selectively to the surface of the plate, or selectively to an antibody specific to that analyte/antigen. After immobilization, a secondary antibody attached to an enzyme is added that subsequently changes colour upon substrate addition. Its high sensitivity provides added advantages over other analytical procedures like HPLC which is essential for the detection of pico or nanomolar concentrations.

System: ELISA was carried out using standard laboratory equipment, plate washer and absorbance detector (BMG Fluostar, Optima, Offenburg, Germany).

#### 2.1.4.3 Ventilator System

Ventilator: mechanical ventilator Puritan Bennett 840 (PB840) (Covidien, Boulder, CO, USA). Airway pressure and flow data were recorded from the ventilator using Matlab (MathWorks, Natick, MA, USA).

#### 2.1.4.4 Global Positioning System and Video-Analysis Systems

GPS system 1: VX Sport, VX330, Wellington, New Zealand

GPS system 2: Catapult minimaxx S4, Victoria, Australia

GPS software: Catapult Sprint version 5.1

Video analysis: Opta provided live performance data analysis of rugby related studies while a Go-Pro<sup>®</sup> videoed the mixed martial arts contests.

### 2.1.5 Solutions and Buffers

#### 2.1.5.1 SCX-HPLC Solutions and Buffers

Strong cation exchange (SCX) HPLC was performed for the detection of neopterin (NP), 7,8-dihydroneopterin, XP, 7,8-dihydroxanthopterin, biopterin (BP), 7,8-dihydrobiopterin (BH<sub>2</sub>) and tetrahydrobiopterin (BH<sub>4</sub>). The analysis was performed on a SCX column (Luna<sup>™</sup> SCX, 100Å, 250 mm x 4.6 mm ID, 5 µM) with a solvent mixture of 20 mM ammonium phosphate (AmPO<sub>4</sub>) pH 2.5 buffer as the mobile phase. The buffer was prepared by dissolving 2.64 g AmPO<sub>4</sub> in 900 mL nanopure H<sub>2</sub>O (Milli Q<sup>™</sup>, Millipore). After balancing the pH meter with both a pH 4 and pH 7.2 solution, the buffer was adjusted dropwise to pH 2.5 with concentrated phosphoric acid. Final volume was made up to 1000 mL with nanopure H<sub>2</sub>O and filtered through a 0.45 µm filter before being sonicated for five minutes.

For the conversion of BH<sub>4</sub> and BH<sub>2</sub> to BP, as well as 7,8-dihydroneopterin to NP, an acidic-tri-iodide and ascorbic acid solution were used. Acidic-tri-iodide was prepared by dissolving 2.7 g solid iodine (I<sub>2</sub>) and 5.4 g potassium iodide (KI) in 35 mL of NANOpure H<sub>2</sub>O in a

measuring cylinder. To this mixture, 4.37 mL of concentrated hydrochloric acid (HCL) (12.08 M) was added in a fume cupboard. The  $I_2$  was completely dissolved using a magnetic stirrer and made up to a final volume of 50 mL with NANOpure  $H_2O$ . An ascorbic acid solution (0.6 M) was prepared fresh daily by dissolving 0.1057 g L-ascorbic acid in 1 mL nanopure  $H_2O$  in a 1.7 mL tube.

All standards for SCX-HPLC analysis were prepared by dissolving 1.5 – 2 mg in the 20 mM  $AmPO_4$  pH 2.5 buffer. Neopterin and creatinine solutions were stored at  $-20^{\circ}C$  in 200  $\mu L$  aliquots. They were thawed and diluted using mobile phase buffer to 25 nM on the day of analysis. All other standards were prepared fresh on the day of analysis using the same protocol.

#### 2.1.5.2 ELISA Buffers

For the “in-house” cortisol and myoglobin ELISA’s, an assay buffer and plate coating buffer were used to dilute samples/block plates and adsorb the primary antibody to the ELISA plate respectively. The assay buffer was prepared in a 10X solution by dissolving 28.76 g  $Na_2HPO_4$ , 6.56 g  $NaH_2PO_4 \cdot H_2O$  and 90 g NaCl in 800 mL nanopure  $H_2O$ . In a separate large beaker, 10 g gelatin was dissolved using a microwave and added to the dissolved buffer salts. 10 mL of Tween 20 was added lastly and made up to 1 L with nanopure  $H_2O$ . A 1X solution was prepared by heating the 10X concentrate in a warm  $H_2O$  bath and adding 100 mL to 900 mL nanopure  $H_2O$  that resulted in a pH 7.4. The coating buffer protocol is identical excluding the addition of Tween 20 and gelatin.

#### 2.1.5.3 Urine Collection for Myoglobin Analysis

For the analysis of myoglobin in urine, a 0.2M NaOH solution was prepared that adjusted the pH of each urine sample to 8 – 9.5. This was prepared by dissolving 8 g NaOH pellets in 1000 mL NANOpure  $H_2O$  in a beaker using a magnetic stirrer and aliquoted into 20 x 50 mL falcon tubes.

## 2.2 METHODS

### 2.2.2 Urine Analysis

Urine samples were collected from each subject using a 70 mL collection pottle. They were asked to provide a specimen mid-stream (urine from the bladder). For all pterin analyses and N-terminal prohormone of brain natriuretic peptide (NT-proBNP) analysis, samples were immediately placed on ice inside a 40 L chilli-bin. For myoglobin analysis, five mL of each urine sample was aliquoted at time of collection into a separate sterile 15 mL falcon tube containing 750  $\mu$ L 0.2M NaOH to adjust the pH to approximately 8 - 9.5 as described previously (de Waard and van't Sant 2009). Following the amateur rugby game, an experiment was conducted with the urine of the subjects that identified the optimal volume of 0.2M NaOH to add to a pre and post-exercise urine sample to adjust the pH to the correct value (Appendix N).

#### 2.2.2.1 Pterin Assay

The pterin assay used in this research utilizes both fluorescence and absorbance. The reason for both forms of detection revolves around the different spectral properties of the pterins in question. Both NP and BP are highly fluorescent allowing for the detection and quantification and their reduced forms following conversion by acidic tri-iodide. The reason for this conversion is based on the minimal fluorescence of these compounds which is similar for creatinine and dihydroxanthopterin. In these cases, absorbance is used to calculate their concentrations.

The SCX chromatography employed utilizes the principle of cation-exchange. For the particular column (Luna<sup>TM</sup> SCX) used here, the stationary phase is made up of negatively charged sulphonyl groups which interact with the positive charge of the analyte of interest at low pH. This low pH keeps the analyte protonated increasing its retention time. This type of analyses can be used for proteins, nucleotides and amino acids whose elution is based on the pH and composition of the mobile phase and column temperature/length/side-chain composition. Total NP (NP + 7,8-dihydroneopterin), total BP (BP + BH<sub>2</sub> + BH<sub>4</sub>) and dihydroxanthopterin were detected at NP's native fluorescence. Although it was optimally set for NP, the highly fluorescent nature of the other compounds made them extremely

detectable and also allowed for the simultaneous determination without having to complete separate time consuming and expensive assays. The detector was set at an excitation wavelength of 353 nm and emission wavelength of 438nm while absorbance for creatinine was set at 234 nm and xanthopterin (XP) at 254nm.

#### *2.2.2.1.1 Urinary Pterin and Creatinine Assay*

For urinary pterin and creatinine quantification, two separate HPLC assays had to be completed. The first involved the oxidation of the dihydro-compounds, while the second did not. For the quantification of NP, BP, XP and creatinine, urine samples were thawed in tap H<sub>2</sub>O, vortexed, and diluted 1 in 40 (25 µL urine was added to 975 µL buffer) with 20 mM AmPO<sub>4</sub> pH 2.5. 100 µL of the diluted sample was aliquoted into the HPLC inserts ready for analysis. For the quantification of total NP and total BP, 20 µL of acidic tri-iodide (5.4 % I<sub>2</sub>/10.8 % KI in 1 M HCL) was added to a separate tube containing 100 µL of the 1 in 40 diluted urine sample. The sample was vortexed briefly and incubated in the dark for 15 minutes. This was followed by the addition of 10 µL 0.6 M ascorbic acid to neutralize the excess acidic tri-iodide in the sample. 100 µL of the sample was added to an insert where 10 µL was injected onto a SCX column (Luna<sup>TM</sup> SCX, 250 mm x 4.6 mm ID, 5µm) maintained at 30°C with a flow rate of 1 mL/min. Fluorescence was set at an excitation wavelength of 353 nm and emission wavelength of 438 nm while the UV detector was set at 234 nm. A 25 nM pterin standard and 100 uM creatinine standard were used to calculate urinary analyte concentrations.

#### *2.2.2.1.2 Experimental Oxidation Experiments*

The detection and quantification of pterins following iron and myoglobin oxidation of 7,8-dihydroneopterin were conducted using the same SCX-HPLC technique.

#### *Concentration Effect*

Concentrations of FeSO<sub>4</sub>-EDTA (0 – 10 mM) and myoglobin (Abcam. Cat. No. ab96036, 0 – 500 µg/mL) were prepared with purified nanopure H<sub>2</sub>O and 20mM AmPO<sub>4</sub> pH 7.4. 10 mM FeSO<sub>4</sub> was prepared by dissolving 2.78 g of iron (II) sulphate heptahydrate in 1000 mL

nanopure H<sub>2</sub>O. 10 mM EDTA was prepared by dissolving 2.92 g of EDTA in nanopure H<sub>2</sub>O. The pH of the solution was continuously adjusted to pH 8 using 5 M NaOH to allow for the complete dissolving of EDTA. 500 µL of each solution was then combined to form a 5 mM FeSO<sub>4</sub>-EDTA complex. 500 µg/mL myoglobin was prepared by diluting a pre-prepared 2.88 mg/mL solution.

50 µL of each concentration was added to 50 µL 20 µM 7,8-dihydroneopterin in a 1.7 mL Eppendorf tube that was freshly prepared, kept on ice and in the dark using the same phosphate buffer. Following vortexing, each sample was incubated at 37°C for two hours. 100 µL of each FeSO<sub>4</sub>-EDTA containing sample were added to an HPLC insert and analysed immediately, while 50 µL acetonitrile (ACN) was added to the myoglobin samples to precipitate the protein which was followed by centrifugation at 13,000 rpm for 10 minutes before HPLC analysis.

#### *Time Course Effect*

50 µL of 100 µM FeSO<sub>4</sub>-EDTA and 500 µg/mL myoglobin were added separately to 20 µM 7,8-dihydroneopterin that were prepared as above. Each sample was mixed and incubated for 0, 15, 30, 60, 120 and 240 minutes at 37°C. Samples containing FeSO<sub>4</sub>-EDTA were analysed immediately, while 50 µL ACN was added to the myoglobin samples to precipitate the protein and centrifugation at 13,000 rpm for 10 minutes before HPLC analysis.

#### 2.2.2.2 Quantification of Myoglobin

A direct sandwich ELISA and RP-HPLC method were used for the quantification of myoglobin in urine. A direct sandwich ELISA combines the use of two antibodies, one to capture and one to detect. Quite often a polyclonal antibody is used to capture as much antigen as possible, while the detection antibody is normally monoclonal and more specific. For this analysis to work, the antigen has to have at least two antigenic epitopes capable of binding to an antibody which provides a very sensitive means of quantification that does not require a purification step.

A 96 well microtitre plate (falcon) was coated overnight with mouse monoclonal antibody to myoglobin (Abcam, Cat. No. ab19607) (5 µg/mL in phosphate buffered saline (PBS), 100

μL/well). Following “blocking” in assay buffer (PBS containing 0.1% gelatin w/v and 0.1% Tween 20 v/v, 150 μL/well), the wells were emptied by inversion. “Blocking” prevents any non-specific binding to the plate from any other compound. Then either 100 μL myoglobin standard (Abcam, Cat. No. ab96036) (1000 to 0.01 ng/mL) diluted in assay buffer, or 100 μL of urine diluted 1 in 2 in assay buffer was added for a 60 minute incubation at 20°C. This was then washed five times automatically with a plate washer and 100 μL/well of rabbit polyclonal antibody (Abcam, Cat. No. ab78841) at 0.5 μg/mL in assay buffer was added for 30 minutes at 20°C. The plate was again washed and goat anti-rabbit IgG-HRP (Jackson ImmunoResearch Laboratories, Cat. No. 111-035-003) (1:2000 in assay buffer) was added for 30 minutes at 20°C. The plates were finally washed and TMB substrate was added (100 μL/well). Colour development was terminated by the addition of 1 M HCl (100 μL/well) and the absorbance read at 450 nm (BMG, Fluostar, Optima).

The reverse phase (RP) chromatography option employs hydrophobicity to separate compounds of interest. Using a polar mobile phase and a hydrophobic stationary phase, compounds adsorb to the stationary phase of the RP column allowing for clear and measureable quantification. By adjusting the concentration of an organic solvent in the mobile phase (ACN or methanol), compounds can be separated precisely without neighbouring interference.

For urinary myoglobin analysis, samples were diluted 1 in 10 with 10 mM C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>.NH<sub>4</sub> pH 7 before 100 μL was transferred to an auto-sampler vial for HPLC analysis. 10 μL of each sample was injected onto a Jupiter C5 5 μm RP 300Å, 150 x 4.6 mm column with mobile phases of H<sub>2</sub>O containing 0.1% TFA (v/v) pH 2.5 (A) and 100% ACN (B). A linear gradient from 10 - 70% B over 15 minutes was followed by 70% B for a further five minutes. A gradient from 70% to 10% B followed over the next two minutes and remained at 10% for 10 minutes making a total run time of 32 minutes. Myoglobin was detected by absorbance at 400 nm. All analytes were quantified by peak area using Shimadzu Class VP software and conducted in duplicate.



### 2.2.2.3 Quantification of N-terminal prohormone of brain natriuretic peptide

Urinary NT-proBNP was measured using double antibody sandwich technology (MyBioSource, Cat No. MBS269035). Urine samples were centrifuged at 3000 RPM for 10 minutes and the supernatant removed for analysis. The plate was pre-coated with NT-proBNP monoclonal antibody. 100 µL of a standard (31.25 – 2000 pg/mL) or 100 µL of an undiluted urine sample were added to each well and incubated at 37°C for 90 minutes. This was followed by automatic plate washing (5X) and the addition of 100 µL/well bio-tinylated polyclonal antibody to NT-proBNP. The plate was further incubated for 60 minutes at 37°C, washed five times, and 100 µL/well horse-radish peroxidase added and incubated at 37°C for 30 minutes. Washing was followed by TMB addition and incubation in the dark at 37°C. Colour development was terminated by the addition of sulphuric acid (100 µL/well) and the absorbance read at 450 nm (BMG, Fluostar, Optima).

### 2.2.2.4 Specific Gravity

Specific gravity was calculated using a hand-held refractometer (N-20, Atago, Tokyo, Japan). 50 µL of each urine sample was added to the refractometer and calculated using distilled H<sub>2</sub>O as a zero standard. Specific gravity (SG) was calculated using the following formula based on the normal population SG<sub>1.020</sub> (Goldberger *et al.*, 1995).

$$2) \quad \text{Concentration}_{\text{SG normalized}} = \text{Concentration}_{\text{specimen}} \cdot (\text{SG}_{1.020} - 1) / (\text{SG}_{\text{specimen}} - 1)$$

### 2.2.3 Saliva Analysis

Whole un-stimulated saliva was collected by passive drool for one minute into a sterile **pre-weighed** 15 mL plastic container with the subject's eyes open, head tilted slightly forward and making minimal orofacial movement. Each subject was asked to rinse their mouth with H<sub>2</sub>O first to remove any food particulate present, and if any blood was present in the sample, they were asked to repeat until clear. All samples were placed on ice immediately after collection and transported back to the laboratory and stored at -80°C until analysis.

### 2.2.3.1 Determination of Saliva Flow

Saliva was weighed to the nearest mg and volume calculated assuming a saliva density of 1.00 g/mL (Cole *et al.*, 1988). The saliva flow rate (mL/min) was determined by multiplying the volume of saliva by the collection time (one min).

### 2.2.3.2 Quantification of Immunoglobulin A

Samples were thawed and an indirect competitive enzyme immunoassay kit (Salimetrics, USA) was used for the quantitative measurement of IgA in saliva samples. Competitive ELISA utilizes a primary antibody in solution with the antigen of interest. Following an incubation period, the solution is then added to a pre-coated plate. The amount of free antibody remaining is inversely proportional to the amount of analyte present in the sample.

On the day of analysis, saliva samples were thawed at room temperature. Goat anti-human salivary IgA conjugated to horseradish peroxidase (diluted 1:120) was added (50  $\mu$ L/tube) to tubes containing specific dilutions of standards (0 – 600  $\mu$ g/mL) or saliva samples that were vortexed and centrifuged at 3000g for 15 min. Each saliva sample was pre-diluted 1 in 5 by adding 25  $\mu$ L of each samples supernatant to 100  $\mu$ L of a sample dilution buffer for the analysis. Each sample was incubated at room temperature for 90 minutes. Subsequent to incubation and mixing, an equal solution (50  $\mu$ L/well) from each tube was added in duplicate to a microtitre plate coated with human salivary IgA. After a 90 minute incubation at 20°C on a plate shaker at 400 rpm, the plate was washed automatically five times followed by the addition of TMB. Colour development was allowed to proceed for a maximum of 30 minutes which was initiated by putting the plate on a plate shaker at 500 rpm for 5 minutes. For the rest of the incubation, the plate was placed in the dark and monitored for blue colour development. The addition of sulphuric acid terminated the reaction and optical density was read on a standard automated plate reader at 450 nm (BMG Fluostar Optima). Saliva IgA secretion rate (ug/min) was calculated by multiplying saliva flow rate by IgA concentration.

### 2.2.3.3 Quantification of Cortisol

Salivary cortisol was measured by competitive ELISA using monoclonal antibodies as described previously (Lewis *et al.*, 1992). On the day of analysis, saliva samples were

thawed at room temperature and centrifuged at 3000 rpm. ELISA plates were coated with cortisol-thyroglobulin conjugate. The following day the plates were washed and “blocked” with assay buffer and then emptied. Standard (0 - 280 nmol/L) or reconstituted saliva extract was added. Saliva (250  $\mu$ L) was extracted with 1.0 mL  $\text{CH}_2\text{Cl}_2$ . After vortexing gently for two minutes, 500  $\mu$ L of each sample was transferred to a separate labelled tube and dried in a heating block (37°C) under air flow until evaporated. The remaining polar compounds were reconstituted in 125  $\mu$ L of assay buffer, vortexed briefly and left to stand for 30 minutes. 50  $\mu$ L of each reconstituted standard or sample was added in duplicate to the pre-coated plate which was promptly followed by an antibody cocktail comprising 1:35 of cortisol monoclonal antibody A2 and 1:1000 of rat anti-mouse IgG1-peroxidase. The plates were incubated for 15 min at 20°C prior to automatic washing (5X). 100  $\mu$ L/well TMB was added with the reaction terminated with 100  $\mu$ L/well 1M sulphuric acid once a distinct blue colour had developed in the standards. Optical density was read on a standard automated plate reader at 450 nm (BMG Fluostar Optima).

#### 2.2.4 Ventilator Analysis

During the experiment, each participant was connected to a mechanical ventilator (Puritan Bennett 840 (PB840) (Covidien, Boulder, CO, USA)) via a facemask tightly fitted to the face. The participant was required to relax and breathe normally with a trained clinician present at the participant’s side to ensure minimal or no leaks from the ventilation circuit. The ventilation mode was set to SPONT to allow spontaneous breathing ventilation triggering.

Ventilator PEEP was initially set to 5 cmH<sub>2</sub>O. At the commencement of the experiment, the participant was required to breathe with ventilator support. After 10 ~ 15 breathing cycles, PEEP was increased to 10 cmH<sub>2</sub>O. The participant was instructed to continue breathing for 10 ~ 15 cycles more. Each participant therefore had 20 ~ 30 breathing cycles for respiratory mechanics estimation.

The time-varying elastance model (Chiew *et al.*, 2011) was used to estimate the respiratory mechanics of the participants.  $E_{drs}$  is the dynamic lung elastance, varying with the time over each breath. With this dynamic parameter, changes in respiratory mechanics within each breath were observed, and thus provided a detailed insight of a player’s lung condition (Chiew *et al.*, 2011). Player-specific respiratory mechanics were defined as:

$$4) \quad P_{aw}(t) = R_{rs}Q(t) + E_{rs}V(t) + P_0$$

Where  $P_{aw}(t)$  is airway pressure,  $t$  is the time,  $R_{rs}$  is the airway resistance,  $Q(t)$  is the flow,  $E_{rs}$  is the respiratory elastance,  $V(t)$  is the air volume entering the lung (tidal volume) and  $P_0$  is the offset pressure. A single breath-specific  $E_{rs}$  and  $R_{rs}$  was determined using a modified multivariate regression method (Chiew *et al.*, 2011). Using  $E_{rs}$  and  $R_{rs}$ , the time varying elastance,  $E_{drs}(t)$  can be determined:

$$5) \quad E_{drs}(t) = (P_{aw}(t) - R_{rs}Q(t) + P_0) / V(t)$$

Airway pressure and flow data were recorded from the ventilator to estimate the participant's-specific  $E_{drs}$  using Matlab (MathWorks, Natick, MA).

### 2.2.5 Global Positioning System

Two separate levels of players were analysed in this study (amateur and professional) which required two separate types of GPS analyses.

The amateur rugby study (study two) used the GPS unit VX330 (VX Sport, Wellington, New Zealand). The unit was placed inside a specifically designed vest worn under the subject's shirt in approximately the upper-thoracic spine region. The total distance covered was split into several velocity speed bands which included passive (0 - 8 km/h), slow (8 - 13 km/h), medium (13 - 18 km/h), fast (18 - 23 km/h) and full (23 - 42 km/h). Devices were turned on 10 minutes before the start of play and turned off immediately following the game. After collection, data were downloaded to a personal computer where further analysis was carried out using the software supplied by the manufacturer.

The professional research (studies three and four) used the GPS unit, Catapult minimaxx S4 (Docklands, Victoria, Australia). Microtechnology was used to quantify time, position and the distance covered in the relevant velocity speed bands at 10 Hz. These consisted of > 7

km/h, > 16 km/h, > 20 km/h and > 25 km/h. Player-load was also calculated at 100 Hz using accelerometers inside the units using the following equation:

$$1) \quad \sqrt{((a_{y1} - a_{y-1})^2 + (a_{x1} - a_{x-1})^2 + (a_{z1} - a_{z-1})^2)}$$

where  $a_y$  = anteroposterior acceleration,  $a_x$  = mediolateral acceleration and  $a_z$  = vertical acceleration. All subjects volunteered to wear the GPS units available. The unit was placed inside a specifically designed vest worn under the subject's shirt in approximately the upper-thoracic spine region. Devices were turned on 10 minutes before the start of play and turned off immediately following the game. After collection, data were downloaded to a personal computer where further analysis was carried out using the software supplied by the manufacturer (Catapult Sprint V5.1).

### 2.2.6 Video-Analysis

For the amateur rugby studies, video-analysis was conducted by myself with players carefully observed through the game duration and number of impacts recorded. An impact was classified when a subject was involved in the following actions; ruck, maul, tackle, hit-up or scrum. Each subject was given a one per situation and tallied up to give overall impacts.

For all professional rugby studies, Opta provided live performance data analysis from each game that was used to calculate the number of impacts experienced by each player. An impact was classified as a tackle or tackle assist, a ball carry resulting in contact with the opposition, and arriving and clearing a ruck on offence or defence. Each subject was given a one per situation and tallied up to give overall impacts.

For the mixed martial arts contests, A Go-Pro® was mounted to the referee's head to give a close up analysis while a separate camera was also placed ringside for a different angle. All contests were individually analysed for the number and type (kick, punch, takedown) of impacts sustained during the contests as previously described (Harriston 2004).

### **2.2.7 Statistical Analysis**

Statistical analysis was conducted using both the statistical package for social sciences version 20 (SPSS), or R version 3.1.1 (Team 2014). Each study has its own separate statistical analysis that is described in the following chapters.



# Study 1

Incorporates method development of a novel high performance liquid chromatography assay capable of quantifying urinary neopterin by strong cation exchange chromatography, and the first clinical experiment of this thesis investigating inflammatory and oxidative stress changes throughout a week of resistance training in competitive natural body-builders.



# Chapter 3

## Measurement of Changes in Urinary Neopterin and Total Neopterin in Body Builders Using SCX HPLC

### Abstract

Body building is a sport where ultrastructural damage to muscle fibres aids the development of dense muscle layers. Using a new SCX based chromatography method to measure neopterin and 7,8-dihydroneopterin, we investigated whether this muscle damage caused increased levels of inflammation during a week of resistance training. Urine samples were collected over eight consecutive mornings from 10 natural competitive body builders. Samples were analysed using SCX HPLC with urine volume corrected for creatinine and specific gravity. The majority of subjects showed large changes in both neopterin and total neopterin (7,8-dihydroneopterin + neopterin) levels, though the mean data for the group showed no significant change over the week. There was no evidence of the high intensity resistance training causing an accumulation of inflammation. The SCX method had an intra- and inter-assay variability of 3.04% and 5.42% respectively. Urine volume correction with SG and creatinine provided near identical results. Creatinine and specific gravity are both reliable methods for correcting for urine volume, SCX HPLC provides a new means of measuring urinary neopterin and total neopterin, and high intensity resistance training causes cumulative inflammation in some individuals.

### 3.1 Introduction

Body-building is a sport where competitors are judged on their muscular physique. Multiple years of preparation and dedication are required for the retention of strict training and nutrition regimes crucial for success. Training typically consists of year round high intensity and high frequency hypertrophy and strength training causing ultra-structural muscle damage through eccentric muscle loading (Gibala *et al.*, 1995; Howatson and Van Someren 2008; Newham *et al.*, 1987; Roth *et al.*, 2000; Staron *et al.*, 1992). Muscle damage results in the invasion of inflammatory cell populations that may last days or sometimes weeks while repair, regeneration and growth occur. A host of cytokines is released into the surrounding area which facilitates the arrival of lymphocytes, macrophages and neutrophils (Calle and Fernandez 2010) and begins the process of regeneration. Other physiological factors such as exercise intensity, oxidative stress, acidosis, heat, intensity, duration, recovery between sets

and training status can influence their secretion rate and concentration (Miles 2008; Petersen and Pedersen 2005; Radom-Aizik *et al.*, 2007). Neutrophils initially invade the damaged muscle which may be phagocytic in nature and help with cellular debris degradation, while macrophages provide several functions including debris removal from an injured muscle which can effect muscle cell differentiation and proliferation (Honda *et al.*, 1990; Lowe *et al.*, 1995; McLennan 1993). Managing the damage, repair and adaption process is critical for continual year-round training and progression. It is a significant concern for body builders who rely on recovery to ultimately build larger, denser muscle and repeat this process on a weekly basis.

The accumulation of repetitive high intensity exercise can potentially lead to an increased susceptibility to infection (Nieman 1996; Nieman *et al.*, 1990), and chronic inflammation (Olson *et al.*, 2007). With common training regimes of five days training and two days off, the persistent eccentric loading could develop the onset of chronic inflammation and immune system activation. Biomarkers such as C-reactive protein (CRP) and interleukin-6 (IL-6) are common markers often used for inflammation detection (Damas *et al.*, 1992; Tomaszewski *et al.*, 2003) and have elevated levels following resistance training (Olson *et al.*, 2007). Identifying the level of potential inflammation accumulation over a week of high intensity resistance training can lead to increasingly effective training protocols to maximize muscle growth and recovery.

#### *Neopterin and 7,8-dihydroneopterin*

Significant attention has been given to CRP, IL-6, interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-8 (IL-8), interleukin-1ra (IL-1ra), interleukin-10 (IL-10), interleukin-15 (IL-15), TNF- $\alpha$  and its soluble receptor (sTNF- $\alpha$ R1) in response to diseases and exercise that elicits an inflammatory response. Neopterin and 7,8-dihydroneopterin are pteridine compounds synthesized and released from activated macrophages stimulated with interferon- $\gamma$  (IFN- $\gamma$ ) (Giese *et al.*, 2008a; Müller *et al.*, 1991; Wachter 1992). They have been elevated in patients with human immunodeficiency virus (HIV) (Fuchs *et al.*, 1989a) and coronary artery disease (Garcia-Moll *et al.*, 2000). GTP-cyclohydrolase, an enzyme upregulated by IFN- $\gamma$ , catalyses the breakdown of GTP to 7,8-dihydroneopterin triphosphate. In primate macrophages, the accumulation results in the diffusion of 7,8-dihydroneopterin out of the activated macrophage and into the intracellular spaces and finally the plasma. Some of the 7,8-

dihydroneopterin is oxidized to the highly fluorescent neopterin (NP) by hypohalous acids such as hypochlorite (Giese *et al.*, 2000, 2001a; Giese *et al.*, 2001b; Widner *et al.*, 2000). The communication of T cells and macrophages and the subsequent release of NP makes it an effective marker of immune system activation and inflammation (Wachter 1992; Wachter *et al.*, 1989) with several studies having identified this rise in plasma and urine following exercise (Gunga *et al.*, 2002; Schobersberger *et al.*, 2006; Sprenger *et al.*, 1992; Tilz *et al.*, 1993). Our thought is the damage to muscle as a result of eccentric loading and hypertrophy training will increase the abundance of macrophages present at these sites, and subsequently produce higher concentrations of 7,8-dihydroneopterin which can be oxidized to NP. Therefore, the high frequency training philosophy of many body builders should elicit an increase in measureable 7,8-dihydroneopterin and NP.

#### *High Performance Liquid Chromatography Analysis*

Due to its high fluorescence, NP is relatively easy to detect in plasma and urine using reverse phase high performance liquid chromatography (HPLC) (Flavall *et al.*, 2008; Fuchs *et al.*, 1983; Giese *et al.*, 2008a), though many clinical laboratories also use ELISA as well (Westermann *et al.*, 2000). Although immunoassays are ideal due to the ease of use and rapid processing time, the immunoassays developed for NP are expensive. Reverse phase separation of urine components for NP analysis is the most commonly reported method used (Wachter *et al.*, 1979) as it is fast, reliable and accurate (Fuchs *et al.*, 1982). We have found the reverse phase methods gives poor separation and resolution of NP from other urinary components. Following advice from Schirks Laboratory in Switzerland, we have developed and report here on an ion exchange based separation method using strong-cation exchange (SCX) HPLC chromatography with ultraviolet (UV) detection at 353nm for excitation and 438nm for emission. Currently a combination of NP and 7,8-dihydroneopterin have only been measured in plasma in relation to atherosclerotic plaque formation (Giese *et al.*, 2000, 2001a) and not exercise. The majority of reports on exercise have focused specifically on urine and plasma NP levels (Fuchs *et al.*, 1982). While this provides information about an acute inflammatory response and level of oxidative stress, it does not provide information about the total inflammatory response. Oxidation of NP from 7,8-dihydroneopterin *in vitro* allows the quantification of total immune system activation inclusive of the level of oxidation present.

*Urine Volume Normalization*

Urine volume normalization is critical for analyte concentration determination. Creatinine and specific gravity (SG) are the two methods currently employed (Fuith *et al.*, 1990; Schulze *et al.*, 2008). The upper limits of normal ( $\mu\text{mol NP/mol creatinine}$ ) for women range as follows: 208 (18-25 years), 209 (26-35 years), 239 (36-45 years), 229 (46-55 years), 249 (56-65 years), 251 (older than 65 years). In men upper limits of normal are slightly lower: 195 (18-25 years), 182 (26-35 years), 176 (36-45 years), 197 (46-55 years), 218 (56-65 years), 229 (older than 65 years). These upper limits include 97.5% of healthy controls (Wachter *et al.*, 1989). It is common practice in medicine to use creatinine as a marker of urine volume correction (Iizuka *et al.*, 1993) as it is released at a fairly constant rate (Shaffer 1908). For doping tests, SG is the choice of the World Anti-Doping Agency (WADA) but it is not widely used due to a lack of data comparing creatinine to SG.

This research aims to provide information pertaining to the potential elevated concentrations of NP and 7,8-dihydroneopterin over the course of a week of high intensity resistance training in competitive natural (no use of banned substances) body building, and to provide an alternative HPLC technique using SCX based chromatography with urine volume correction using both creatinine and SG.

**3.2 Methods***Subjects*

Ten healthy controls with an average age ( $\pm\text{SD}$ ) of  $32 \pm 8$  years, height of  $181 \pm 8.1$  cm and weight of  $80.6 \pm 6.7$  kg, and eight competitive natural (no pharmaceutical enhancements) body builders that train and compete in Christchurch and New Zealand volunteered for this study. The characteristics of the subjects enrolled in the study, including their experience in the sport and training phase are in Table 3.1.

The experimental protocol was approved by the University of Canterbury Human Ethics Committee, Christchurch, New Zealand and all subjects were informed (Appendix B and C) of the risks involved in the study before their written consent was obtained (Appendix A)

Each subject trained one-two body parts per day, three-six exercises per body part, three-six sets per exercise and eight-twelve reps per set. All subjects took a range of nutritional supplements that were not monitored due to the large variation and quantity of products taken, and all were of satisfactory health during and after the study based on a questionnaire (Appendices D and E).

**Table 3.1.** Subject characteristics.

Subject	Age	Height (m)	Weight (kg)	Phase	Calories/day	Experience
<b>S1</b>	25	1.68	70.0	Mass gain	2000	Novice
<b>S2</b>	46	1.70	92.0	Mass gain	3500	Novice
<b>S3</b>	25	1.77	77.1	Competition prep	1900	Multiple shows
<b>S4</b>	22	1.68	76.0	Mass gain	2000	1 show
<b>S5</b>	26	1.91	88.0	Competition prep	2500	Novice
<b>S6</b>	23	1.74	80.0	Mass gain	4000	Novice
<b>S7</b>	34	1.63	58.5	Competition prep	1300	Multiple shows
<b>S8</b>	19	1.78	79.0	Competition prep	2500	Novice
<b>Mean</b>	27.5	1.74	77.6		2462	
<b>SD</b>	8.6	0.09	10.3		889	

### *Experimental Design*

Inclusion criteria required subjects to be a natural competitive body builder with a training schedule of five days on/two days off. Control subjects provided a single urine sample for comparison. Each body-building subject was provided with eight urine canisters and asked to provide a sample mid-stream upon the second time they needed to pass urine each morning. The sample was either frozen immediately at the subject's home or stored in an ice-filled cooler box provided. The first sample was collected on the morning after their scheduled two days of rest, and each morning for the following seven mornings. Samples were collected at the cessation of the eight days and transported to the laboratory, aliquoted into four 1.7 mL centrifuge tubes per sample, and frozen at -80°C until analysis.

*Sample Preparation, HPLC Analysis and Specific Gravity*

Refer to Chapter 2.2 (Materials and Methods).

*Precision Studies and Recovery*

Intra-assay precision was evaluated using 20 replicates of a single urine sample in a single analytical run spiked with 50 and 500 nmol/L NP standard. Inter-assay precision was evaluated using 20 replicates of a single urine sample on four consecutive days for both concentrations. The variation was calculated using the following equation.

$$3) \quad \%CV = (SD_{\text{samples}} / \text{Mean}_{\text{samples}}) \times 100$$

A calibration curve was established using NP and creatinine standards. Solutions of 10, 50, 100, 500 and 1000 nM pterin and creatinine concentrations were prepared on the day of analysis and run in triplicate. Areas under the curve were used to plot a standard curve and repeated in triplicate.

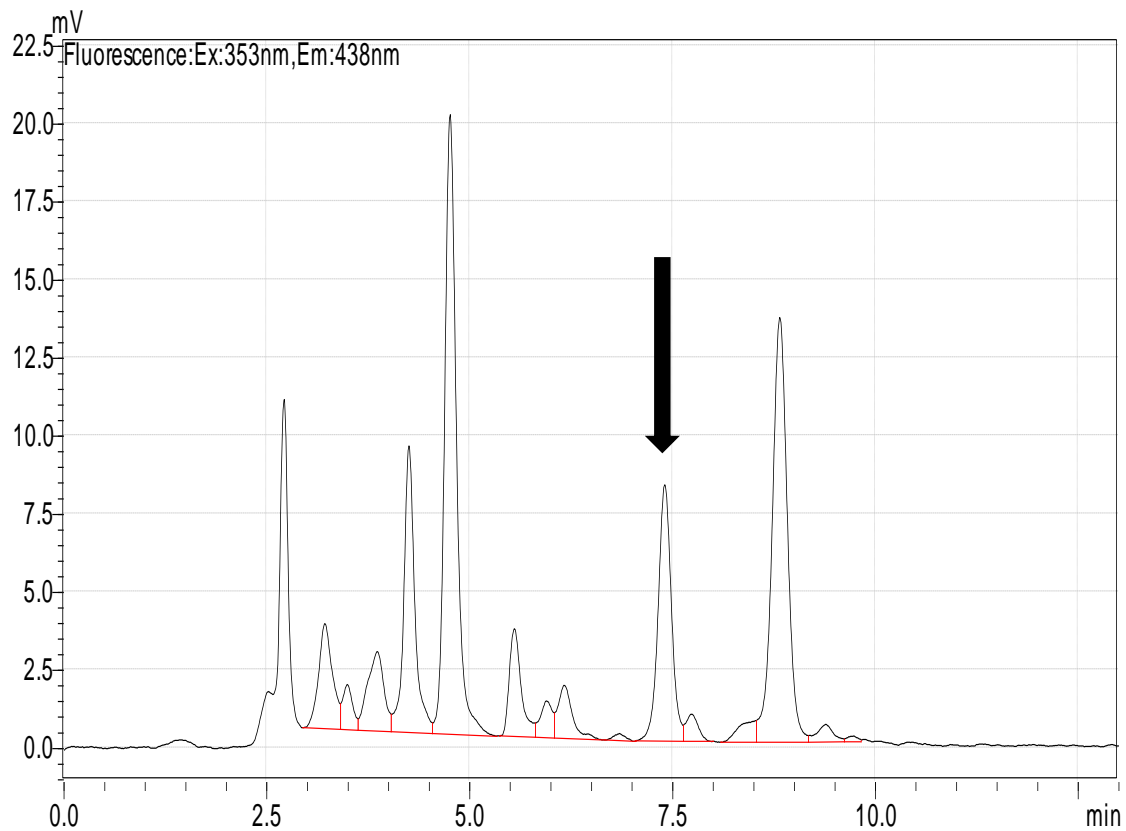
Spiking and recovery of standard and urine samples with a known quantity of NP was also conducted. Both 50 and 500 nM NP were added to a 100 nM NP standard and urine sample. Using LC solutions software, peak areas were calculated to ascertain the recovery of the known addition.

*Statistical Analysis*

Data was analysed using the statistical package for social sciences (SPSS) version 20. A repeated measures analysis of variance (ANOVA) was conducted to investigate changes in urinary NP and total NP corrected for creatinine and SG during the week of body-building training.

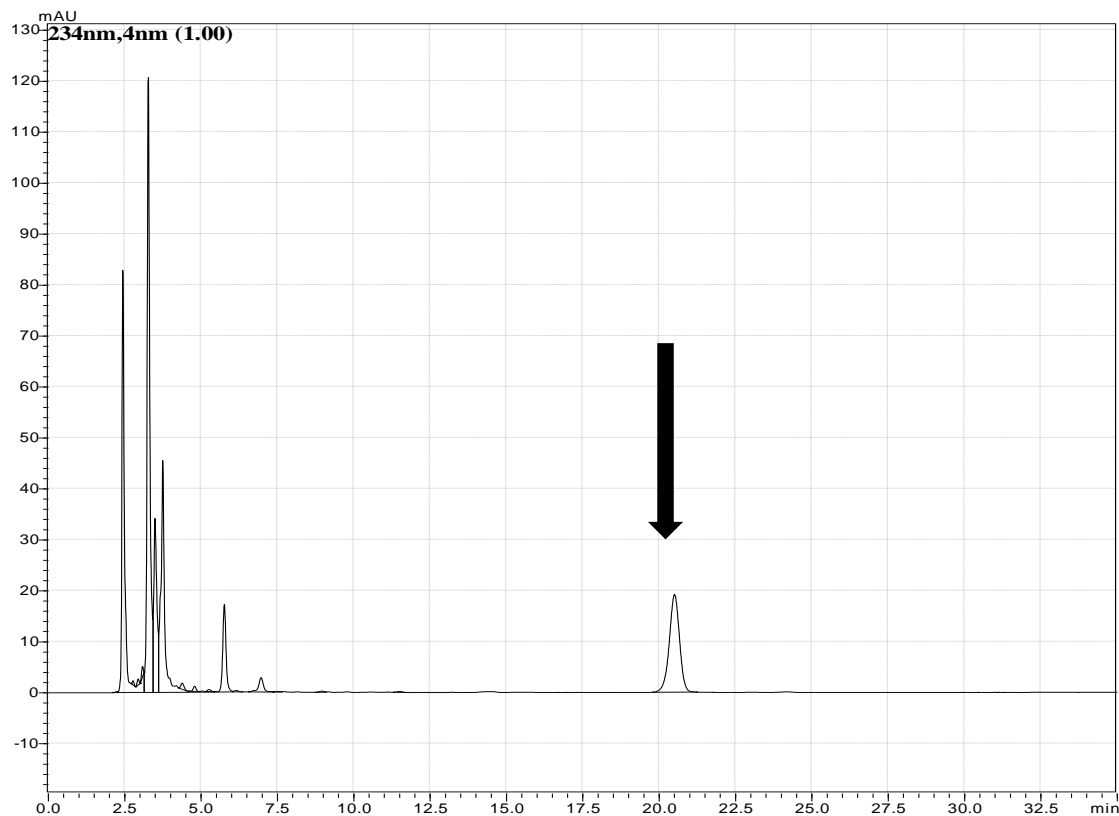
**3.3 Results**

Urinary NP and creatinine were detected in the same HPLC run eluting at 7.4 and 20.4 minutes respectively (Fig. 3.1 and 3.2). Each peak stands alone, is clearly visible and sharp with no visible tailing. The intra-assay coefficient of variation for NP was 3.04 % while the inter-assay coefficient of variation was 5.42 %. Over the range of standards (25-1000 nmol/L<sup>-1</sup>), the assay presented a linear response.



**Figure 3.1.** SCX chromatography analysis of NP in a control subject's urine. Neopterin was detected by its native fluorescence (Ex 353nm Em 438nm) at 7.4 minutes as indicated by the arrow.

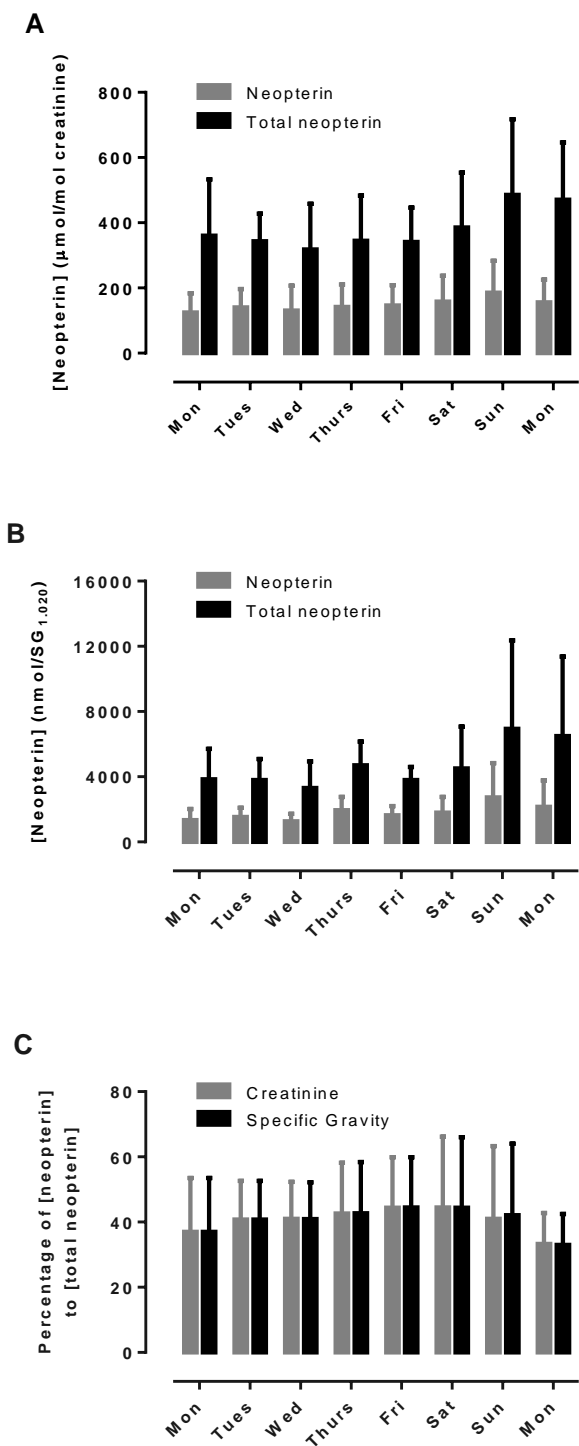
Control subject values for NP/CR, total NP/CR, NP/SG and total NP/SG are presented in Table 3.2. There was no statistically significant change in the concentration of NP or total NP from baseline during the week of training for the body builders when corrected for creatinine (Fig. 3.3A) or SG (Fig. 3.3B). There is a trend towards an increasing inflammatory response toward the end of the training week which is observed with both creatinine and SG correction.



**Figure 3.2.** Creatinine elution from the same control subject’s urine shown in figure 3.1. HPLC conditions remained consistent, however this chromatograph represents spectrophotometer analysis. The chromatograph shows the absorbance of creatinine at 234 nm eluting at 20.4 minutes.

There were significant differences ( $p > 0.05$ ) when comparing the body-building group to controls on certain days for NP/CR and every day for total NP/CR and total NP/SG (Table 3.2). All NP/CR values remained within the upper limits of normal (Wachter *et al.*, 1989) for the healthy population, however some subjects had values as high as 360  $\mu\text{mol/mol}$  creatinine.





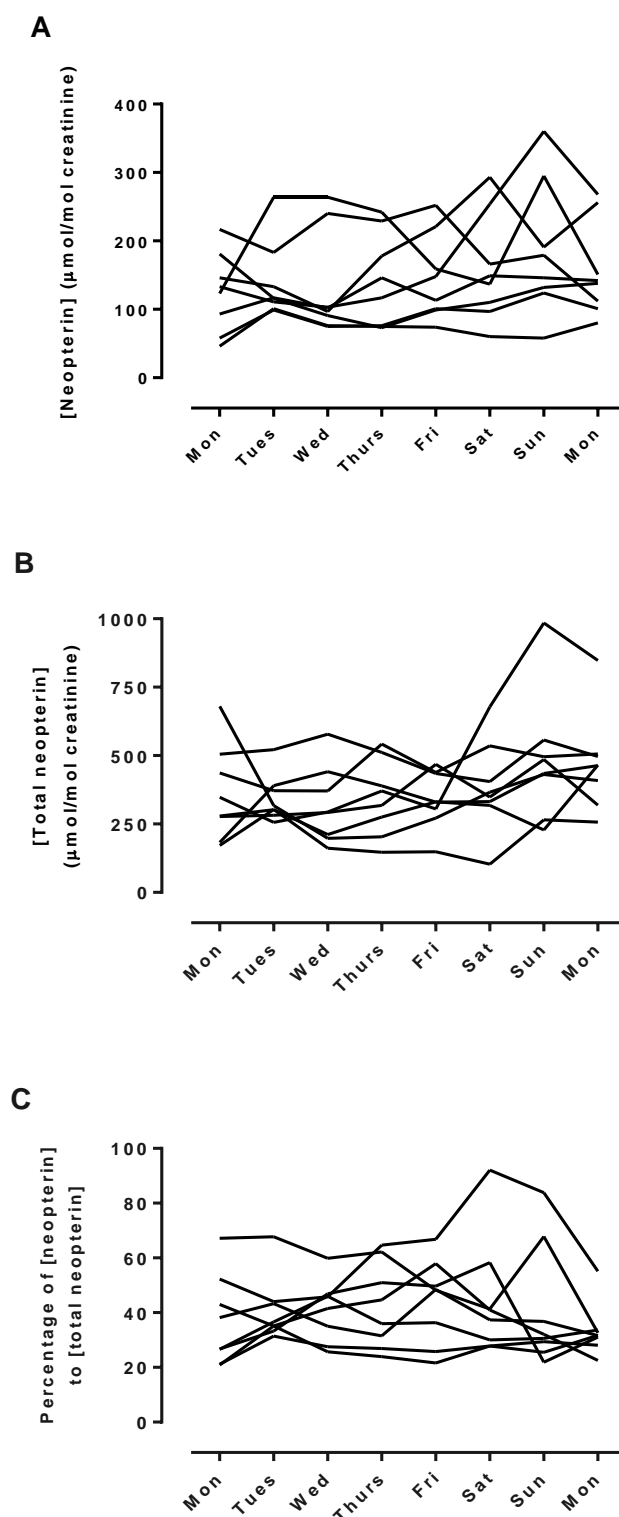
**Figure 3.3.** Mean urinary NP and total NP concentrations for the group during a regular week of resistance training. The data shows NP and total NP (NP + 7,8-dihydroneopterin) corrected with creatinine (A) or SG (B) and a comparison between creatinine and SG on the percentage of NP to total NP (C). Data is presented as mean  $\pm$  SD.

**Table 3.2.** Control subjects (C) and mean daily body building subjects NP and total NP concentrations corrected with creatinine and SG. Values are mean  $\pm$  SD. \* denotes statistically different from control concentrations,  $p < 0.05$ .

Day	NP/CR ( $\mu\text{mol/mol}$ )	TNP/CR ( $\mu\text{mol/mol}$ )	NP/SG ( $\text{nmol/SG}_{1.020}$ )	TNP/SG ( $\text{nmol/SG}_{1.020}$ )
C	93 $\pm$ 39	175 $\pm$ 64	1233 $\pm$ 681	2234 $\pm$ 1028
1	124 $\pm$ 58	360 $\pm$ 173*	1345 $\pm$ 673	3840 $\pm$ 1864*
2	140 $\pm$ 56*	343 $\pm$ 84*	1530 $\pm$ 566	3793 $\pm$ 1294*
3	131 $\pm$ 75	318 $\pm$ 139*	1263 $\pm$ 467	3308 $\pm$ 1630*
4	142 $\pm$ 68	344 $\pm$ 138*	1946 $\pm$ 818	4699 $\pm$ 1449*
5	145 $\pm$ 62*	341 $\pm$ 105*	1636 $\pm$ 562	3794 $\pm$ 808*
6	158 $\pm$ 79*	385 $\pm$ 168*	1803 $\pm$ 958	4508 $\pm$ 2563*
7	185 $\pm$ 79*	485 $\pm$ 231*	2729 $\pm$ 2106	6944 $\pm$ 5413*
8	156 $\pm$ 69*	470 $\pm$ 176*	2160 $\pm$ 1607	6493 $\pm$ 4878*

The percentage of 7,8-dihydroneopterin oxidized to NP did not significantly change for creatinine or SG during the training week (Fig. 3.3C). Each urine volume correction method demonstrated near identical values, while percentage values varied significantly among subjects ( $p < 0.01$ ) with values ranging from 20.9 - 92.1%.

There is a large individual variation in NP and total NP secretion between subjects (Fig. 3.4). Within this variation, there is a general trend toward an increasing NP and total NP concentration for creatinine and SG correction, while the level of oxidation is quite random. Although some subjects demonstrate greater increases compared to other, some show little day to day variation.



**Figure 3.4.** Urinary NP (A), total NP (B) and percentage of 7,8-dihydroneopterin oxidized to NP (C) corrected with creatinine for each individual bodybuilder.

### 3.4 Discussion

The method commonly employed for urinary NP detection is based on the reverse phase method developed in 1979 (Wachter *et al.*, 1979). de Castro et al (2004) developed a similar reverse phase method with an intra- and inter-assay co-efficient of variation (CV) of 12.9 % and 12.5 % respectively. With an intra- and inter-assay CV of 3.04 % and 5.42 % respectively, the described SCX ion exchange method provides a similarly reliable, repeatable and reproducible method that allows for the simultaneous detection of both urinary NP and creatinine. Control subject concentrations are similar to the normal range of urinary NP in healthy individuals (Wachter *et al.*, 1989) demonstrating its reliability for providing an alternative to reverse phase chromatography. Additionally, it is a simple and effective quantification method that provides simultaneous quantification of NP and creatinine with no neighbouring interference (Fig. 3.1 and 3.2.).

This study reports urinary NP concentrations whilst comparing urine volume correction methodologies. Whilst numerous drugs are SG corrected (Cone *et al.*, 2009), the nearly identical values observed when correcting NP with creatinine and SG (Fig. 3.3A, 3.3B and 3.4C) provide evidence of a reliable alternative method for urine volume normalization. The removal of the need to measure creatinine drastically reduces chromatographic run times for NP analysis and simultaneously decreases the chance of false-negative results following exercise that is known to increase creatinine concentrations by as much as 50 - 100% (Anderson *et al.*, 1982; Decombaz *et al.*, 1979). Moreover, this correction does seem to be suitable for both high intensity and endurance exercise (WADA Technical Document, 2004).

Although the size of the study cohort resulted in a lack of statistical power there was an evident trend as a result of this research. There is a noticeable increase during the training week for NP and total NP concentrations corrected with creatinine and SG that suggests there may be an accumulation of inflammation and oxidative stress as a result of five days intense resistance training (Fig. 3.3A and B.). This may represent signs of under-recovery and subsequent functional overreaching (FO), non-functional overreaching (NFO) or even overtraining syndrome (OTS) in extreme circumstances. This information might be crucial for some individuals to tailor their training specifically around their needs rather than conforming to what is considered the “best” or “norm” for developing muscle.

While there is a noticeable trend, NP/CR values fall within the normal reference range (Wachter *et al.*, 1989) indicating conclusions cannot be drawn in terms of inflammation and immune system activation during a week of competitive natural body building training. Contrasting evidence however, identifies “normal” as  $294.6 \pm 178.6$   $\mu\text{mol/mol}$  creatinine (de Castro *et al.*, 2004) indicating more research may be required to understand the “normal” level using different analytical methods. This of course is based on the assumption that neopterin does not have day-to-day variation. Total NP/CR, NP/SG and total NP/SG values however, are significantly higher than control subjects (Table. 3.2) suggesting the training regime of a competitive natural body builder may be sufficient enough to cause the individual to be in a continual state of immune system activation and toward the upper end of “normal” using this particular HPLC method.

Assumptions pertaining to the relevance of NP concentrations should consider whether NP or total NP has been measured. Since 7,8-dihydroneopterin oxidation is solely dependent on the oxidative status of the individual, the total NP/CR and total NP/SG may provide a more effective representation of the total inflammatory response that is not altered by ROS. The extreme inter-subject percentage variability of NP compared to total NP (20.9 – 92.1%) provides clear evidence of the potential errors associated with this determination considering previous evidence has suggested a 7,8-dihydroneopterin – NP ratio of 3:1 (Fuchs *et al.*, 1989b). While some individuals in this study abide by this, the majority do not. It therefore suggests the combination of 7,8-dihydroneopterin and NP provides the most accurate assessment of macrophage activation. With statistical differences observed only between the body-building and control groups for total NP, two conclusions may be drawn. Oxidative status does not change during a week of resistance training, but it may elicit a significant inflammatory event as a consequence of the ultra-structural muscle damage.

Sample collection is an important aspect regarding accurate dissemination of immune system activation. Resistance training has been shown to elicit muscle damage and provoke an inflammatory reaction as measured by several cytokines (Petersen and Pedersen 2005). The current study identified a lack of statistical change throughout the week of training suggesting no one day stimulated the inflammatory response more than another. Previous studies have identified immediate increases in plasma NP and other inflammatory cytokines post-exercise (Nieman *et al.*, 2004; Peake *et al.*, 2006; Schobersberger *et al.*, 2006) suggesting the delay in

sample collection of the current study may have masked the full extent of total immune system activation. With concentrations significantly greater than the control group, it does suggest the intensity was sufficient enough to cause structural integrity loss of the muscle cell membrane, but due to a possible adaptive response to the training, concentrations returned to the “normal” range. Similar responses have been postulated in chronic inflammatory diseases where the use of resistance training decreases the level of circulating inflammatory biomarkers (Beavers *et al.*, 2010). One study which investigated resistance training over a one year period found a reduction in plasma CRP (Olson *et al.*, 2007) whilst several reviews have stated that tumor necrosis factor-alpha (TNF- $\alpha$ ) does not change in response to resistance training when CRP decreases (Ploeger *et al.*, 2009). Furthermore, the 24 hours between training sessions may be sufficient enough to allow the repair process to be complete and thus inflammation becoming undetectable.

The research to date remains equivocal with long term resistance training and its effects on physiological and biochemical markers of stress. Whilst some use traditional resistance training of two-three days a week at 80% 1RM to monitor inflammation (Stewart *et al.*, 2007), sampling procedures and days training remain diverse. Some studies have measured before and after a training session (Peake *et al.*, 2005) whilst others have used a seven week training period followed by an acute bout of resistance exercise to differentiate any changes in stress markers (Izquierdo *et al.*, 2009). These approaches are not in accordance with common body building or weight lifting protocols and thus cannot be used to definitively define the stress accompanied by commonly used training programs. Studies must differentiate the foci of the research between an acute or chronic exercise focus in order to produce a clear and relevant approach to data collection and analysis. This study measured the chronic exposure to high intensity resistance training and subsequently allowed the observation of delayed onset muscle soreness and chronic inflammation. With values reaching their peak in the days of rest, it is indicative of cumulative inflammatory response and compounding macrophage activation.

The inter-individual variation observed in this research (Fig. 3.4) highlights the importance of separating research based on group or population data. Although no significant differences between days of training or rest when mean values for the group were compared, specific patterns were observed for certain individuals. Based on questionnaires gathered from each

subject, none presented any cold or flu symptoms, nor did they report any symptoms during the week following training. This suggests the values reported are indeed a true representation of the training week and can imply that this training results in a cumulative inflammation response in certain individuals. Some individuals however, seem to cope with the intensity and can subsequently continue with their current training regime without hindrance to recovery or an over-activated immune system. It has to be noted, that total workload was not measured which may be responsible for the inter-individual variability; although volume of eccentric resistance training does not necessarily translate directly to degree of muscle damage and associated inflammation (Paschalis *et al.* 2005).

The level of NP throughout the week (Fig. 3.3C and 3.4C) would suggest oxidative “status” remained unchanged indicating resistance training of this nature does not induce an increased flux of free radicals. Individually, there were significant differences between subjects which further highlights the need to monitor the individual and their unique response. It is difficult to ascertain whether 7,8-dihydroneopterin production or ROS generation is the limiting factor in its oxidation to NP based on the lack of change in NP or total NP in a group and individual context. The source of plasma and urinary NP is poorly understood considering hydrogen peroxide and other ROS generate dihydroxanthopterin from 7,8-dihydroneopterin. Hypochlorite which is generated by activated macrophages and neutrophils during an inflammatory response is the only known compound capable of oxidising 7,8-dihydroneopterin to NP *in vivo* (Gieseg *et al.*, 2000; Gieseg *et al.*, 2001b; Schraufstätter *et al.*, 1990; Widner *et al.*, 2000). So though it is likely that there are other mechanisms for NP generation during inflammation, they have yet to be described.

### *Conclusion*

A new, reliable and repeatable method for simultaneous detection of urinary NP and creatinine has been demonstrated using ion exchange SCX analytical chromatography which can be used for subsequent rugby and MMA related studies. Neopterin and 7,8-dihydroneopterin are sensitive markers of immune system activation during a week of high intensity resistance training in body builders. The training shows a trend toward increasing levels of inflammation even though values are within the upper limits of normal. Healthiness and the individual response of each subject should be meticulously considered. Creatinine and SG were found to be both reliable and effective means of correcting for urine volume.





# Study 2

Separated into two chapters involving the same cohort of elite amateur rugby players recruited from within the Premier Division of Christchurch rugby. Individual and group time course changes of selected biomarkers are examined following a selected game in the first chapter, whilst the second chapter examines the efficacy of ventilation as an alternative mechanism for identifying lung inflammation/damage.

# Chapter 4

## Changes in Acute Biochemical Markers of Inflammatory and Structural Stress in Rugby Union

### Abstract

Rugby union is a sport governed by high force and frequency impacts. Analysis of physiological markers following a game can provide an understanding of the physiological response of an individual, and the time course changes in response to recovery. Urine and saliva were collected from 11 elite amateur rugby players 24 hours before, immediately after, and at 17, 25, 38, 62 and 86 hours post-game. Myoglobin, salivary immunoglobulin A, and cortisol were analysed by ELISA while neopterin and total neopterin were analysed by high performance liquid chromatography. There was a significant post game increase of all markers except sIgA which remained unchanged. Cortisol increased 4-fold, myoglobin 2.85-fold, neopterin 1.75-fold and total neopterin 2.3-fold when corrected with specific gravity. All significant changes occurred post-game only, with markers returning to and remaining at baseline within 17 hours. The intensity of the game caused significant changes in key physiological markers of stress. They provide an understanding of the stress experienced during a single game of rugby and provide the time course changes associated with player recovery. Neopterin provides a new marker of acute inflammatory response detection in physical exercise while specific gravity should be considered for urine volume correction post-exercise.

### 4.1 Introduction

Rugby union is a sport governed by high force and frequency impacts played in 100 countries across five continents (Quarrie and Hopkins 2007). There has been an increase in intensity since its emergence as a professional sport (1995) with tackle forces as high as 1.95-2.31 x bodyweight having been reported (Usman *et al.*, 2011) with as much as 166 tackles per hour in the professional game (McIntosh *et al.*, 2010). This intensity change has resulted in a decrease in work to rest ratios from 1:20 (Duthie *et al.*, 2005) to 1:5 (Austin *et al.*, 2011) and an increase in injury prevalence (Eaves and Hughes 2003).

High intensity running and impacts commonly associated with rugby union have been shown to cause significant changes in markers of muscle damage, inflammation, psychophysiological stress and immune system function (Cunniffe *et al.*, 2011; Cunniffe *et al.*, 2010; Smart *et al.*, 2008; Takarada 2003; Thompson *et al.*, 1999). Significant attention has been paid to the increase of myoglobin that leaks into the plasma following structural integrity loss of the muscle cell membrane as a result of eccentric loading or blunt force trauma. It has been used extensively as a marker of muscle damage in rugby (Takarada 2003), downhill running (Sorichter *et al.*, 1997), cycling (Suzuki *et al.*, 1999), and patients with rhabdomyolysis (Feinfeld *et al.*, 1992) or acute kidney injury (Zager and Burkhart 1997).

Selective biomarkers of an acute phase inflammatory response demonstrate similar patterns following a rugby game. Interleukin-6 has been shown to rise immediately, while C-reactive protein (CRP) significantly increases above pre-game levels 14 and 38 hours later (Cunniffe *et al.*, 2010). Neopterin, an oxidation product of 7,8-dihydroneopterin produced by interferon- $\gamma$  (IFN- $\gamma$ ) stimulated macrophages, is a marker of cellular immune system activation, ratio indicator of associated oxidative stress and indicative of a pro-inflammatory immune status. It is commonly shown to increase in patients with HIV (Fuchs *et al.*, 1989a) coeliac's disease (Fuchs *et al.*, 1983) and allograft recipients (Aulitzky *et al.*, 1988) and rise in response to different exercise models (Schobersberger *et al.*, 2006; Sprenger *et al.*, 1992). With its use in measuring inflammation in the clinical field well understood, it should therefore provide relevant inflammatory information pertaining to the trauma of a rugby game. Similarly, changes in circulating concentrations of immune system parameters have also been highlighted (Cunniffe *et al.*, 2010) with high incidence rates of infection having been reported in individuals with salivary immunoglobulin A (sIgA) deficiency (Hanson *et al.*, 1983) or low saliva flow rates (Fox 1985). Whilst exercise of a moderate intensity promotes resistance to upper respiratory tract infections, severe exertion coupled with mental stress causes athletes to be vulnerable to subclinical and clinical infection (Pedersen and Toft 2000). A combination of decreased sIgA and increased cortisol, the principal glucocorticoid which has been widely used as a marker of psychophysiological stress in exercise (Chavez 2009; Farrell *et al.*, 1983; Fry and Lohnes 2010), can represent a good measure of stress and respiratory illness in rugby players (Cunniffe *et al.*, 2011).

Traditionally the physical demands of rugby union have been assessed through game analysis (Roberts *et al.*, 2008), injury analysis (Brooks *et al.*, 2005) or from questionnaire analysis for signs of player fatigue (Cresswell and Eklund 2005). The use of selected biochemical markers of “stress” can provide the physiological response of a player over time, irrespective of the “workload”, and potentially be used to justify when an individual is best suited to re-enter training. The focus should be on the treatment of an individual player to distinguish the variability in response to stress. Typically data is analysed and displayed as mean or median data of a team or group. While this provides an overall understanding of the “treatment” on the group, it does not distinguish between responders and non-responders. This becomes particularly important in rugby where players can experience different levels of stress depending on a number of factors that include distance run and number of impacts experienced. This research will analyse both group and individual data to highlight how important it is to not assume everyone experiences the same type of “stress” and subsequent response.

The purpose of this study is to identify the sensitivity, level and time course changes of selected markers of muscle damage, inflammation, psychophysiological stress and immune system function in a non-invasive and stress free manner following an elite amateur rugby game that may be used to manage player recovery.

## 4.2 Methods

### *Participants*

Eleven rugby players from two senior men’s division one rugby teams volunteered for the study. Details of participants enrolled in the study shown in Table 4.1. The experimental protocol was approved by the University of Canterbury Human Ethics Committee, Christchurch, New Zealand and all participants were informed (Appendix G) of the risks involved in the study before their written consent was obtained (Appendix F).

*Experimental protocol and sample collection*

Urine and saliva samples were collected 24 hours pre-game, immediately post-game and at 17, 25, 38, 62 and 86 hours post-game. Each subject was asked to provide a urine sample mid-stream and a saliva sample at each time point. They were also asked to fill out a post-game recovery questionnaire (Appendix H) and health questionnaire (Appendix I) to ascertain their recovery protocols post-game and fitness level for participation respectively.

*Urine and saliva collection*

Refer to Materials and Methods (Chapter 2.2).

**Table 4.1.** Subject characteristics.

<b>Rugby Position</b>	<b>Height (m)</b>	<b>Weight (kg)</b>
Back	1.81	90
Back	1.88	97
Back	1.82	85
Back	1.87	87
Forward	1.89	93
Forward	1.87	122
Forward	1.88	100
Forward	1.78	96
Forward	1.95	102
Forward	1.96	112
Forward	1.81	88
<b>Median [IQR*]</b>	1.87 [1.81-1.89]	96 [88.5-101.5]

\* *Inter-quartile range*

*Game statistics and Sample Analysis*

Refer to Materials and Methods (Chapter 2.2)

*Statistical Analysis*

Analysis was completed using the statistical package for social sciences (SPSS) version 20. All data is presented as mean  $\pm$  SD and analysed using repeated measures analysis of variance (ANOVA) and Spearman's rank correlation. A Bonferroni test was used for post-hoc analysis comparison of means.

**5.3 Results**

The total number of impacts for the group was  $46 \pm 25$  while  $6029 \pm 690$  total metres were covered on average consisting of  $47.0 \pm 7.4\%$  passive,  $27.7 \pm 5.3\%$  slow,  $17.4 \pm 7.1\%$  medium,  $6.0 \pm 3.1\%$  fast and  $1.7 \pm 1.4\%$  full.

Pre to post-game urine pH decreased significantly ( $p = 0.002$ ) from  $6.33 \pm 0.6$  to  $5.67 \pm 0.36$  respectively. There was a statistically significant difference ( $p < 0.05$ ,  $n^2_{\text{partial}} = 0.353$ ) in the group mean urinary myoglobin concentration ( $6.2 \pm 5.7$  to  $17.5 \pm 12.6$  ng/mL) immediately post-game compared to 24 hours pre-game (Fig. 4.1A). Concentrations returned to baseline within 17 hours ( $6.8 \pm 6.9$  ng/mL) and remained at baseline until 86 hours (Fig. 4.1A). Similar results were observed for cortisol (Fig. 4.1B).

Immediate post-game salivary cortisol concentrations significantly increased ( $p = 0.002$ ,  $n^2_{\text{partial}} = 0.583$ ) from 24 hours pre-game to post-game ( $15.2 \pm 7.2$  to  $60.5 \pm 24.6$   $\mu\text{mol/L}$ ) and returned to baseline within 17 hours (Fig. 4.1B) and remained so until 86 hours.

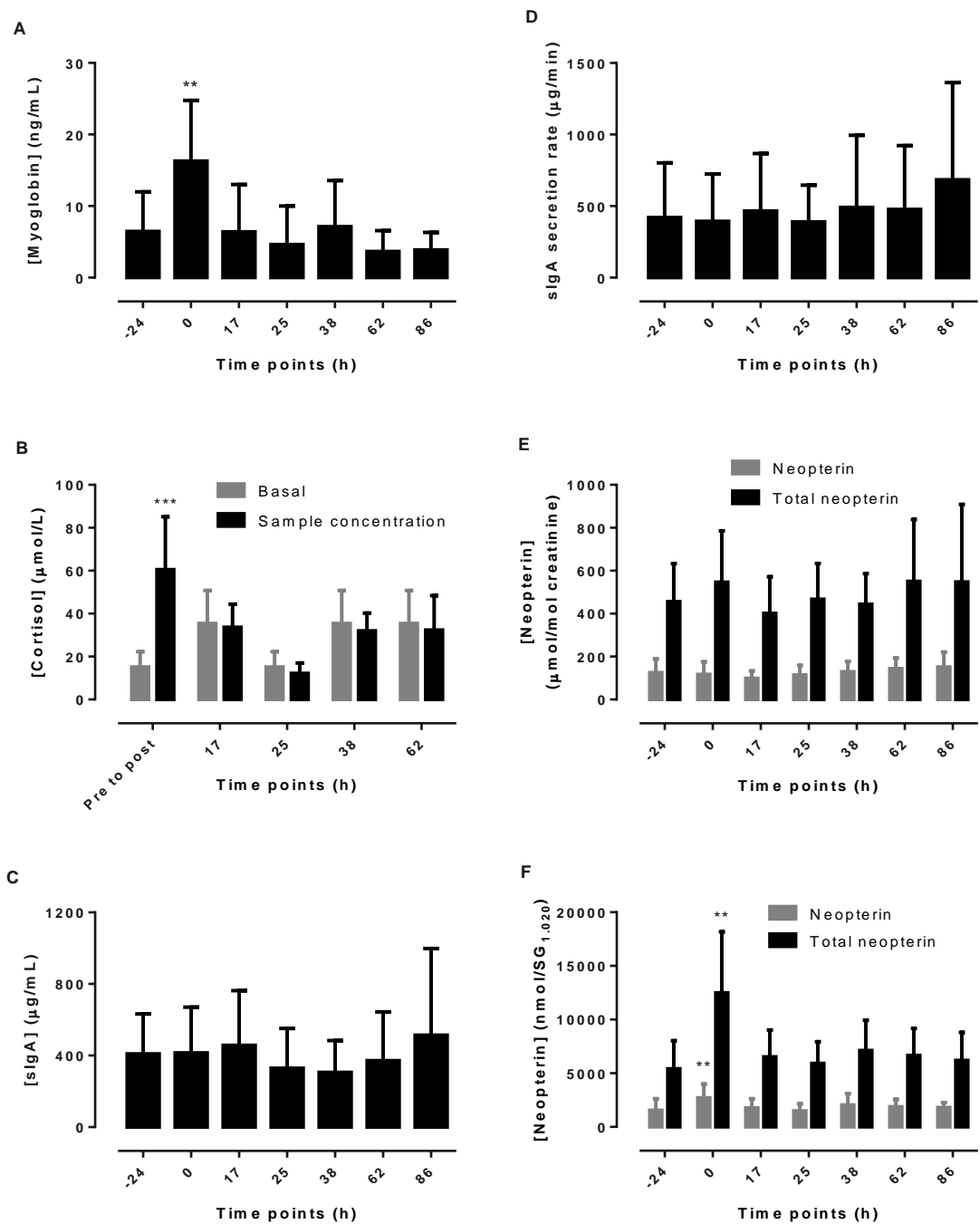
sIgA concentration ( $409 \pm 223$  to  $414 \pm 255$   $\mu\text{g/mL}$ ) and secretion rate ( $419 \pm 383$  to  $394 \pm 330$   $\mu\text{g/min}$ ) did not change in response to the rugby game between pre-game and post-game (Fig. 4.1C and D.) while neopterin (NP) and total NP concentrations when corrected for creatinine showed no significant change as well (Fig. 4.1E.). When urine volume was corrected with specific gravity (SG), NP ( $p = 0.02$ ,  $n^2_{\text{partial}} = 0.315$ ) and total NP ( $p = 0.008$ ,  $n^2_{\text{partial}} = 0.625$ ) were significantly increased immediately post-game compared to pre-game and returned to baseline within 17 hours and remained there for 86 hours (Fig. 4.1F).

The averaged data shown in figure 4.1 masks how individual players are responding to the stress of the game. Examination of the data showed that for all but a few players, each marker peaked immediately after the game and decreased to pre-game levels by 17 hours and

remaining there for up to 86 hours. For simplicity, four representative subjects of the group were chosen to represent the time course changes of total NP and myoglobin, two key markers of physical damage (Fig. 4.2). For subjects where no change was observed post-game, no subsequent rise was observed at later time points (data not shown). The analysis showed that stress was best measured immediately post game.

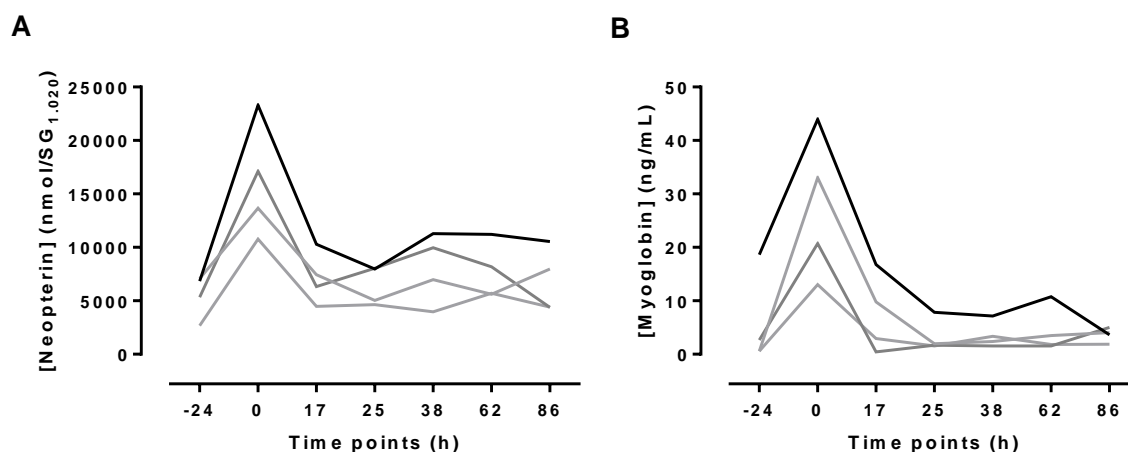
There were large individual variations in response to the game stress when examined immediately post game (Fig. 4.3). The majority of myoglobin concentrations increased pre to post-game (Fig. 4.3A), while cortisol concentrations all increased but with observably different magnitudes (Fig. 5.3B). sIgA concentration (Fig. 4.3C) and secretion rates (Fig. 4.3D) all showed variable changes in response to the game, while NP/specific gravity (SG) (Fig. 4.3E) and total NP/SG (Fig. 4.3F) all increased (except for one subject) pre to post-game with observable differences seen in the pre and post-game concentrations between participants.

Statistically significant correlations found between the biochemical markers of stress and video and global positioning system (GPS) analysis are highlighted in bold in Table 4.2. There were significant negative correlations found between NP/SG with cortisol, sIgA concentration, and distance ( $p < 0.05$ ), and total NP/SG with distance ( $p < 0.05$ ), and a positive correlation between total NP/SG with total impacts ( $p < 0.05$ ).



**Figure 4.1.** Time course changes of urinary myoglobin (A), salivary cortisol (B), sIgA concentration (C), sIgA secretion rate (D), urinary NP/CR and TNP/CR (E) and urinary NP/SG and TNP/SG (F). Basal salivary cortisol is represented by saliva collected before and after the collection protocols at the corresponding times of day. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

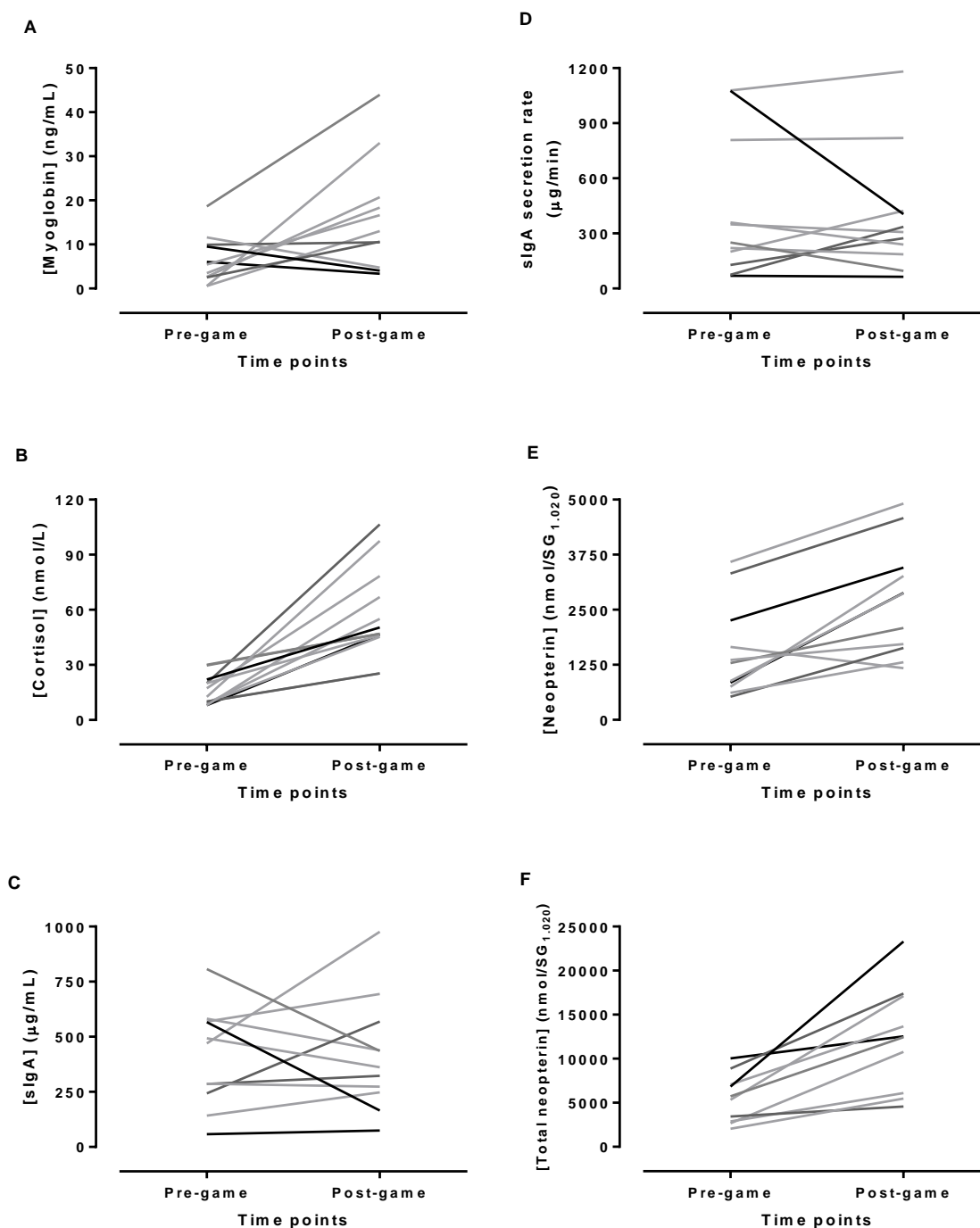




**Figure 4.2.** Time course changes of urinary myoglobin (A) and urinary TNP/SG (B) for selected individuals showing the return to baseline within 17 hours of the game.

**Table 4.2.** Correlations between biochemical markers and video and GPS analysis. Correlations are presented as p and cc (correlation coefficient) values with bold and  $p < 0.05$  representing a significance.  $N = 11$  unless otherwise stated.  $^+$  denotes a positive correlation and  $^-$  denotes a negative correlation.

	Distance run (n=7)	No. of impacts	Mb	Cortisol	sIgA secretion rate	sIgA	NP/SG
No. of impacts	0.878	-	-	-	-	-	-
Mb	0.819	0.718	-	-	-	-	-
Cortisol	0.432	0.698	0.873	-	-	-	-
sIgA secretion rate	0.482	0.059	1.000	0.467	-	-	-
sIgA	0.071	0.915	0.272	0.051	0.125	-	-
NP/SG	<b>0.014<sup>-</sup></b> (cc=0.29)	0.915	0.729	<b>0.011<sup>-</sup></b> (cc=0.28)	0.417	<b>0.016<sup>-</sup></b> (cc=0.31)	-
TNP/SG	<b>0.036<sup>-</sup></b> (cc=0.37)	<b>0.014<sup>+</sup></b> (cc=0.34)	0.385	0.235	0.555	0.235	0.096



**Figure 4.3.** Urinary myoglobin (A), salivary cortisol (B), sIgA concentration (C), sIgA secretion rate (D), urinary NP/SG (E) and urinary TNP/SG (F) pre to post-game response of each subject.

## 5.4 Discussion

The selected markers of “stress” are sensitive and capable of scientifically identifying the physiological response of a player following a rugby game. Individual analysis (Fig. 4.3) shows a large variable response which provides further evidence to the unique use of biochemical analysis in monitoring a player. It highlights why it is important not to rely on group data as number of impacts and distance covered is so player specific, and why managing players as individuals would be the most appropriate when it comes to recovery. Furthermore, the time course changes of biochemical markers can be used to monitor the recovery progression of individuals that reduces the error of subjective and qualitative assessment. When the biological markers return to, or fall below pre-game levels, it could provide additional information about the physiological recovery from the game stress. Seasonal recovery could be monitored in such ways by comparing levels to that of baseline before a season commences to understand how a player is faring as a result of the continual week by week play.

This study provides the first evidence that NP and total NP produced as a result of a rugby game dominated by physical contact, can be used a sensitive marker of inflammation, immune system activation and oxidative stress. The significant increase from pre-game to post-game, return to baseline within 17 hours, and a positive correlation with impacts observed in the acute phase inflammatory response (Fig. 4.1F and Table 4.2) when SG correction was applied is in accordance with other studies examining inflammation in rugby (Cunniffe *et al.*, 2010) and rugby league (Mendham *et al.*, 2012). This is indicative of an immediate activation of cells of monocyte lineage producing 7,8-dihydroneopterin, but not enough to cause a sustained inflammatory effect (Fig. 4.1F). A return to baseline may indicate a player has recovered from the stress of a game with no further immune system activation.

When creatinine correction is applied, the most commonly used form of urine normalization, it would suggest there is no inflammatory response with concentrations falling within the normal reference limit (Wachter *et al.*, 1989). Whilst creatinine and SG are good predictors of hydration status (Miller *et al.*, 2004), a game of rugby significantly increases urinary creatinine ( $p = 0.02$ ), increasing the chance of false-negative results, and therefore losing any potential meaningful significance with a urinary analyte. The significant elevation in urine

acidity provides further information pertaining to metabolic acidosis/anaerobic metabolism and the difficulty associated with urine correction. It would seem logical to correct urine volume with SG following exercise which is the current practice by the World Anti-Doping Agency (WADA) (WADA Technical Document. 2004), however careful consideration has to be given to the hydration status of samples with a SG < 1.003 and > 1.030 (Alessio *et al.*, 1985). This range has been suggested to exclude “diluted” and “concentrated” samples, however others have used ranges as low as 1.002 for example (Cone *et al.*, 2009). Two samples within this study fell outside of this range, 1.0326 and 1.0318, but in our opinion, do not alter the results of the analysis.

The increase in NP from pre-game to post-game (Fig. 4.1F and 4.3E) provides further information concerning the change in oxidative status as a consequence of a rugby game. Oxidative stress, defined as “a disturbance in the pro-oxidant-antioxidant balance in favour of the former” (Sies *et al.*, 1985), is most likely a result of skeletal muscle contraction (Davies *et al.*, 1982a). Homeostatic disruption can cause free radical induced muscle force reduction and increased muscular fatigue through their effects on the sarcoplasmic reticulum (Coombes *et al.*, 2002; Matuszczak *et al.*, 2005). Hypochlorite is the only oxidant *in vivo* capable of oxidizing 7,8-dihydroneopterin to NP at a high yield (Gieseg *et al.*, 2000; Gieseg *et al.*, 2001b; Schraufstätter *et al.*, 1990; Widner *et al.*, 2000). Hypochlorite is released by activated neutrophils during immune system activation and appears to be responsible for some of the oxidative damage during inflammation (Halliwell and Gutteridge 1999). There is continued discussion about whether its concentration limits the conversion or whether the concentration of 7,8-dihydroneopterin is the limiting factor. The significant increase in NP/SG suggests there is an increase in hypochlorite that could potentially decrease skeletal muscle force production and impede recovery.

The time course changes for myoglobin (Fig. 4.1A) are consistent with current knowledge of its elimination kinetics (Suzuki *et al.*, 1999) and time to peak (Mikkelsen and Toft 2005). These make it a sensitive and useful marker for quantifying the extent of acute muscle damage following a rugby game. The large inter-individual variation (Fig. 4.3A) highlights the variability of muscle damage and the importance of managing each player separately. Expected values were significantly lower in comparison to other exercise induced muscle damage protocols (maximum of 44.4 ng/mL compared to 410 µg/mL (Smith 1968)).

Differences in concentration may be due to the relative instability of myoglobin in urine and the time to analysis (seven days after collection). Temperature, pH, unidentified urinary compounds smaller than 10kDa, time to analysis, short half-life, metabolism to nephrotoxic compounds and dissociation at acidic pH have all been identified as causes for myoglobin instability (Alterman *et al.*, 2007; Chen-Levy *et al.*, 2005; Gabow *et al.*, 1982; King *et al.*, 2010; Naka *et al.*, 2005; Wu *et al.*, 1994). A reddish-brown colour was present in some samples suggesting the presence of heme, a state that only occurs when 100 mg/dL is present (Huerta-Alardín *et al.*, 2005) and suggestive of a much higher concentrations as a result of the blunt force trauma commonly associated with rugby union than calculated. We suggest urine samples be analysed immediately after collection if possible to rule out any degradation. In addition, the minimal correlation ( $r = 0.19$ ) between impacts is in disagreement with previous work. This may be due to sample size, type or time to assay (Smart *et al.*, 2008; Takarada 2003), or rather the force, angle or body part involved in the impacts that is the determining factor influencing the concentration of muscle damage markers.

Cortisol had similar response (Fig. 4.1B). The individual variability and acute change (Fig. 5B) make it a sensitive marker for assessing acute psychophysiological stress which is in accordance with previous studies (Elloumi *et al.*, 2003). The four fold increase however, potentially due to the intensity of the game, was twice as large. In addition, its use as a marker of recovery progression has been studied in international rugby (Cunniffe 2012). While this research showed a continued elevation 14 hours post-game and a decrease below baseline levels at 38 hours, this study did not. The difference might be attributable to intensity differences, whilst the elevation may impede recovery and the dip below baseline may represent a rebound anabolic stimulus. This evidence suggests monitoring of salivary cortisol could be used to understand player recovery and when is the best time to re-enter training in preparation for the following game.

The lack of change in sIgA concentration and secretion rate (Fig. 4.1C and D) is in accordance with previous studies (Koch *et al.*, 2007), suggesting this specific game of elite amateur rugby was of an intensity that did not cause immune system suppression. On an individual level, (Fig. 4.3C and D), analysis identified a suppression in some subjects which suggests those players are in an immune compromised state that can result in an increased

susceptibility to infection. Coupled with an increased cortisol concentration, this could inhibit the recovery of a player which has been highlighted in previous work (Cunniffe *et al.*, 2011; Neville *et al.*, 2008). The return to pre-game concentrations could be used in conjunction with cortisol and NP to distinguish the recovery progression of an individual. These individual variations may be attributable to fluid intake (Nehlsen-Cannarella *et al.*, 2000), synthesis, transportation and/or secretion differences between subjects (Reid *et al.*, 2001).

Whilst other correlations exist between some markers, it is difficult to conclude this is meaningful due to sample size and the chance of a type one error. The negative correlation found between NP/SG and total NP/SG with distance covered would be expected to be reversed, whilst the similar correlation between NP/SG and cortisol and NP/SG and sIgA concentration suggests the higher the level of stress and immune system function, the lower the level of inflammation, which when compared to previous research would seem doubtful.

### *Conclusion*

An elite amateur rugby game elicits significant changes for the group in biochemical markers of muscle damage, psychophysiological stress and inflammation with no change in immune system status. These markers provide a physiological snapshot about the stress encountered by a single game of rugby that can be used in conjunction with questionnaires and game analysis to monitor acute stress and recovery progression. It is evidently clear each individual responds differently and it is imperative they be treated this way. Neopterin provides a new and sensitive method of detecting an acute inflammatory response in physical exercise, while SG should be considered for urine volume correction post-exercise.

### *Limitations*

This study is limited by its sample size ( $n = 11$ ) which should be taken into consideration about drawn conclusions.

# Chapter 5

## Lung Injury and Respiratory Mechanics in Rugby Union

### Abstract

Rugby is a popular team contact sport in New Zealand associated with high injury rates. Specifically, there is a chance of inducing internal lung injuries as a result of the physical nature of the game. Such injuries are only identified with the use of specific invasive protocols or equipment. This study presents a model-based method to assess respiratory mechanics of N=11 rugby players that underwent a low intensity experimental mechanical ventilation (MV) test before and after a rugby game. Subjects were connected to a ventilator via a facemask and their respiratory mechanics estimated using a time-varying elastance model. All subjects had a respiratory elastance  $<10 \text{ cmH}_2\text{O/L}$  with little difference observed between pre and post-game respiratory mechanics ( $P>0.05$ ). Model-based respiratory mechanics estimation has been used widely in the treatment of the critically ill in intensive care. However, the application of a ventilator to assess the respiratory mechanics of a healthy human beings is limited. This is the first study to conceptualise the assessment of respiratory mechanics in healthy athletes as a means to monitor post exercise stress and therefore manage recovery.

### 5.1 Introduction

Rugby is an intense, physically demanding contact sport. The physical stress encountered in a single game of rugby is associated with a high risk of injury (Kaplan *et al.*, 2008) due to the increased development of larger, faster and stronger players. Since its emergence as a professional sport in 1995, tackle forces have are as high as  $1.95\text{-}2.31 \times \text{bodyweight (kg)}$  (Usman *et al.*, 2011) which increases the chances of injury in the 80 minute duration of a game.

During the course of a rugby game, players can average as much as 6715 running metres (Coughlan *et al.*, 2011) which includes work to rest ratios as high as 1:5 in a game, which suggests the importance of measuring (Austin *et al.*, 2011) such stress. Additionally, the intermittent nature of rugby consists of 37% walking, 27% jogging, 14% striding, 5% high intensity running and 6% sprinting (Cunniffe *et al.*, 2009). In conjunction with breathing frequencies as high as 62 breaths/min and tidal volumes rising as high as  $3.29 \text{ L}^{-1}$  during

maximal exercise (Clark *et al.*, 1983), respiratory mechanics assessment for lung injury and inflammation is an important aspect in the overall picture of a players health.

One of the major body parts at risk of contact injury is the trunk (Kaplan *et al.*, 2008). Trunk injuries such as hematoma, bruises and fracture can be diagnosed through simple physical examination and other biological markers, however assessment as to the extent of any internal injuries cannot be made through superficial examination. For instance, there may be changes to respiratory mechanics through lung injuries occurring due to player collisions around the trunk region during a game (Borghi-Silva *et al.*, 2008) which can be hard to diagnose. With as many as 166 tackles per hour in the modern game (McIntosh *et al.*, 2010), there is clearly a risk of changes in respiratory mechanics due to lung injury, however these cannot currently be identified without the use of detailed radiographic imaging (Hayashi *et al.*, 2013). Given the highly invasive nature of such techniques, the assessment of lung injury is often neglected because of risk of radiation exposure, a dearth of non-invasive alternatives and perhaps due to the belief that such injuries are of relatively minor medical significance (Schmidt 2012). This negligence potentially reduces subsequent rugby player performance and recovery, and potentially exposes them to the risk of long term harm if they are poorly monitored (Smith 2011).

Identifying the extent of an injury or stress during a game of rugby is a very important factor in maximising player performance and recovery. Current methods include investigation of markers of muscle damage (Takarada 2003), inflammation, psychophysiological stress (Cunniffe *et al.*, 2010) and immune system function (Cunniffe *et al.*, 2011). The analysis and interpretation of injury biomarkers can also provide significant factual information. Combined with medical imaging and player questionnaires, these methods enable coaching, training and medical staff to manage a player's recovery and their eventual return to playing and training. To our knowledge, there is no research on the effects of exercise on lung elastance as a measure of respiratory mechanics. In the case of the critically ill, patient-specific respiratory mechanics are assessed via information gathered by a ventilator to aid clinical decision making (Chase *et al.*, 2011). Ventilators are sophisticated and sensitive devices designed to provide breathing support to critically ill patients. As such, ventilators have the inbuilt capability to capture high frequency, accurate, patient specific pressure and flow information (Chiew *et al.*, 2011). Comparatively, the commonly used spirometer for



assessing lung function measures only flow information. As such, the use of a ventilator to capture player specific respiratory mechanics information has the potential to allow for effective and non-invasive assessment of a players respiratory condition.”

Critically ill patients are assessed through specialised protocols (Oostveen *et al.*, 2003) or model-based methods (Steimle *et al.*, 2011). Model-based methods are increasingly popular as they provide a unique insight of a patient’s condition and respond to treatment without the need of specialised or additionally invasive protocols. Nothing currently to our knowledge is known about whether these model-based methods can be used to assess the respiratory mechanics of healthy individuals. The aims of this study were therefore to examine whether the use of a ventilator and model-based methods could be adapted to assess the respiratory mechanics of a healthy individual, and to test this theory on a group of healthy rugby players’ before and after an elite rugby game that could potentially also be used as a new means of evaluating “stress” and implementing recovery strategies to maximise performance.

## 5.2 Methods

Eleven rugby players from two senior men’s division one rugby teams volunteered for the study. Players are identified by position as forwards and backs. The experimental protocol was approved by the University of Canterbury Human Ethics Committee, Christchurch, New Zealand and all participants were informed (Appendix G) of the risks involved in the study before their written consent was obtained (Appendix F).

Participant characteristics, game statistics, including estimated total lung capacity (TLC) are shown in Table 5.1. TLC is estimated using the regression equations of the European Respiratory Society (ERS) (Ouwens *et al.*, 2002) defined:

$$6) \quad \text{Total Lung Capacity} = 7.99 * \text{Height} - 7.08$$

This study used a model-based method to assess the respiratory mechanics of rugby players, pre- and post- game. The injuries acquired during a rugby game or any collision sport may be attributed to capillary shear-stress failure. This injury has been documented in rugby players

as pulmonary oedema and develops due to extreme pulmonary hypertension, which acts upon a relatively “unprotected or non-adapted” lung (Levy *et al.*, 1996; Lively and Stone 2006; Smith 2011). Therefore, changes in respiratory elastance could detect early stress failure. This failure occurs in players who are able to produce short, high and intense energy bursts with a confounding effect of repetitive physical impacts. The method in this study potentially allows these changes to be detected. This manuscript presents an example of a unique concept study to detect respiratory mechanics changes in healthy athletes using a minimally invasive breathing test.

**Table 5.1.** Participant information and game statistic for the rugby game

Player Identifier	Rugby Position	Height (m)	Weight (kg)	Total Lung Capacity (L)	Number of Impacts	Distance Travelled (m)
R1	Forward	1.78	96	7.14	63	-
R2	Back	1.88	97	7.94	17	5542
R3	Forward	1.96	112	8.58	54	5726
R4	Back	1.81	90	7.38	18	6186
R5	Forward	1.89	93	8.02	69	-
R6	Forward	1.87	122	7.86	69	-
R7	Forward	1.88	100	7.94	61	6575
R8	Back	1.87	87	7.86	4	-
R9	Forward	1.95	102	8.50	54	6679
R10	Back	1.82	85	7.46	28	6652
R11	Forward	1.81	88	7.38	71	4848
<b>Median</b>		1.87	96	7.86	54	6186
<b>[IQR*]</b>		[1.81-1.89]	[88.5-101.5]	[7.40-8.00]	[20.5-67.5]	[5588-6633]+

\*IQR - Interquartile Range; +Median [IQR] for 7 Participants.

### *Ventilator and GPS Analysis*

Refer to Materials and Methods (Chapter 2.2).

Twenty-four hours prior to the game, each participant reported to the intensive care unit (ICU) at St George's Hospital, Christchurch, New Zealand. Prior to the commencement of the protocol, each participant underwent a short training session to familiarize themselves with the experimental apparatus and to reduce the level of stress. The experimental protocol was conducted 24 hours before the game and at 17 hours post-game to observe any differences in respiratory mechanics. These time points were selected to minimize disruption to the pre- and post- game routines of the participants.

Participant-specific  $E_{drs}$  trajectories were compared pre- and post-game at each PEEP level. Participant-specific  $E_{drs}$  was only analysed during the inspiration portion of the breathing cycle. Each breathing cycle was normalised to their total inspiratory time to provide clarity and to ensure consistency between breaths with different inspiratory times. Thus, each cycle effectively began at 0 % and ended at 100 % of the breath-specific total inspiratory time.

For each participant and PEEP, the 5<sup>th</sup>, 25<sup>th</sup>, 50<sup>th</sup>, 75<sup>th</sup> and 95<sup>th</sup> percentile of all  $E_{drs}$  trajectories are presented the results section. The area under the curve of these  $E_{drs}$  trajectories was calculated as  $AUCE_{drs}$ . In each case the  $AUCE_{drs}$  for a normalised  $E_{drs}$  trajectory is thus a surrogate of the participant's respiratory elastance (Chiew *et al.*, 2011). A non-parametric Wilcoxon Rank-sum test was used to test significance. P-values less than 0.05 will be considered to indicate statistically significant (differences in) results.

### 5.3 Results

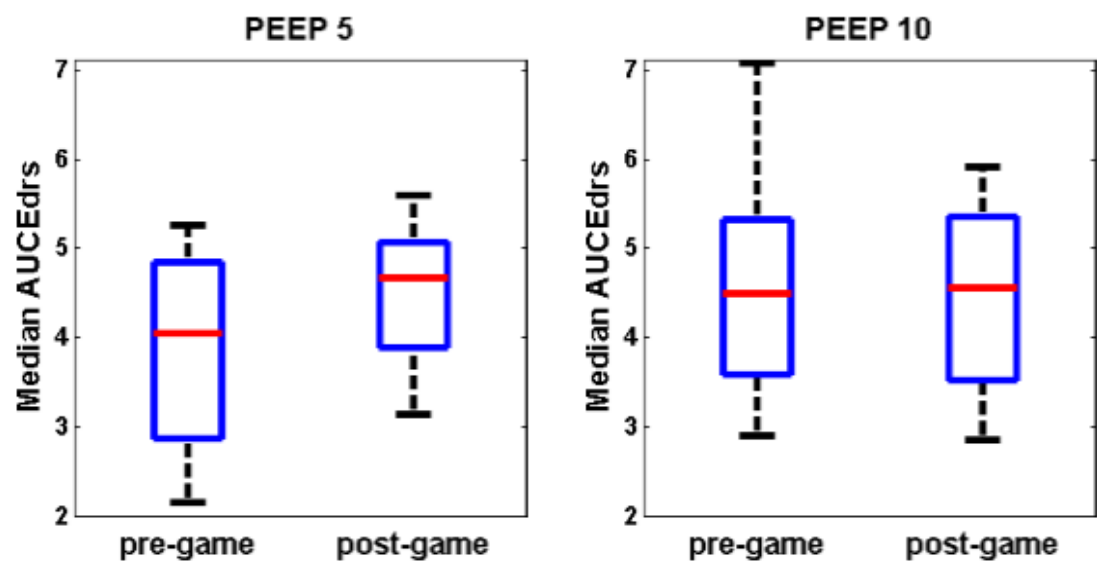
Table 6.2 shows the  $AUCE_{drs}$  for each participant pre and post-game at PEEP 5 cmH<sub>2</sub>O and 10 cmH<sub>2</sub>O. The median [Interquartile range (IQR)] of  $AUCE_{drs}$  was calculated for all participants pre and post-game, and separately for the backs and forwards. The median  $AUCE_{drs}$  found for this study cohort was < 10 cmH<sub>2</sub>O/L, which corresponds to healthy respiratory elastance.

The median [IQR] of  $AUCE_{drs}$  for participants pre-games was 4.0 cmH<sub>2</sub>O/L [IQR: 2.9-4.9] at PEEP 5 cmH<sub>2</sub>O and 4.5 cmH<sub>2</sub>O/L [IQR: 3.6-5.3] PEEP 10 cmH<sub>2</sub>O. The median [IQR] of  $AUCE_{drs}$  for players post-games were 4.7 cmH<sub>2</sub>O/L [IQR: 3.9-5.1] at PEEP 5 cmH<sub>2</sub>O and 4.6 cmH<sub>2</sub>O/L [IQR: 3.5-5.4] PEEP 10 cmH<sub>2</sub>O. Box-and-whisker plots for  $AUCE_{drs}$  are presented in figure 6.1.

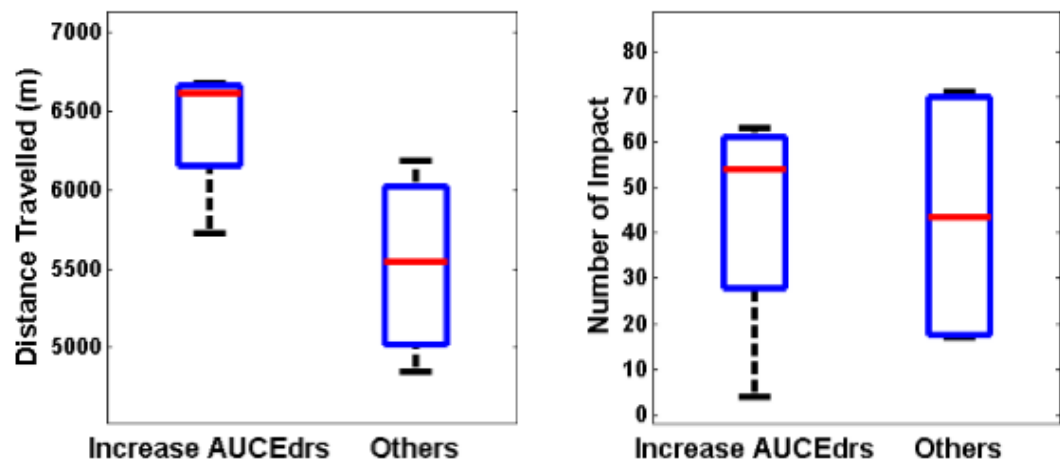
**Table 5.2.** Pre- and post- game respiratory elastance ( $AUCE_{drs}$ ) of each participant at PEEP 5 and 10

Player (Position)	$AUCE_{drs}$ for the 50 <sup>th</sup> [25 <sup>th</sup> -75 <sup>th</sup> ] (cmH <sub>2</sub> O/L)				
	PEEP 5 cmH <sub>2</sub> O			PEEP 10 cmH <sub>2</sub> O	
	Pre	Post		Pre	Post
<b>R1 (F)</b>	4.4 [3.4-5.7]	5.1 [4.0-6.7]	+	5.4 [4.4-7.3]	5.2 [2.7-7.2]
<b>R2 (B)</b>	4.0 [3.1-4.9]	3.3 [1.3-4.5]		5.6 [3.4-9.2]	2.9 [0.9-6.1]
<b>R3 (F)</b>	2.2 [0.5-10.0]	5.2 [3.4-6.6]	+	3.1 [1.6-4.6]	3.5 [1.9-4.8]
<b>R4 (B)</b>	5.3 [4.4-6.3]	4.9 [2.5-6.1]		4.7 [2.2-7.0]	4.8 [2.8-6.2]
<b>R5 (F)</b>	5.0 [4.1-6.1]	4.0 [3.1-5.0]		3.5 [1.9-6.4]	3.5 [2.4-4.6]
<b>R6 (F)</b>	5.0 [3.6-6.8]	5.6 [3.8-6.9]	+	7.1 [5.4-8.8]	5.7 [4.1-6.8]
<b>R7 (F)</b>	2.6 [1.2-3.8]	3.8 [2.4-5.3]	+	2.9 [-2.0-2.9]	4.6 [2.7-6.0]
<b>R8 (B)</b>	3.4 [2.3-4.4]	4.7 [3.1-6.0]	+	4.5 [2.5-6.6]	4.0 [2.9-4.9]
<b>R9 (F)</b>	2.7 [2.0-3.1]	4.6 [2.8-5.7]	+	3.8 [2.2-4.7]	5.9 [3.3-7.8]
<b>R10 (B)</b>	4.0 [1.1-6.5]	4.7 [2.8-6.0]	+	4.5 [3.2-5.6]	5.4 [3.6-6.5]
<b>R11 (F)</b>	4.3 [3.5-5.1]	3.1 [2.4-3.5]		5.0 [4.4-6.3]	3.2 [1.8-12.1]
<b>Overall</b>	4.0 [2.8-4.8]	4.7 [3.9-5.1]		4.5 [3.8-5.0]	4.6 [3.5-5.2]
<b>Forward</b>	4.3 [2.6-4.8]	4.6 [3.9-5.2]		3.8 [3.2-5.3]	4.6 [3.5-5.6]
<b>Backs</b>	4.0 [3.7-4.7]	4.7 [4.0-4.8]		4.6 [4.5-5.1]	4.4 [3.4-5.1]

+ Participant with post-game  $AUCE_{drs}$  increase



**Figure 5.1.** Box-and-whisker plots comparing  $AUCE_{drs}$  pre-game and post-game at each PEEP level for all participants. (Left) PEEP 5 cmH<sub>2</sub>O (Right) PEEP 10 cmH<sub>2</sub>O.



**Figure 5.2.** Separating participants with increase in  $AUCE_{drs}$  and others. (Left) Distance travelled by the participant: (Right) Number of impacts observed.

Figure 5.2 summarises the distance travelled and impacts of participants during the game as recorded by global positioning system (GPS) and video-analysis. Figures 5.3 and 5.4 reveal the  $E_{drs}$  trajectories for two participants (R1 and R9). Participant R1 had relatively little increase in  $AUCE_{drs}$  when comparing pre-game to post-game values at both PEEPs, where participant R9 had higher increase in  $AUCE_{drs}$ .

When comparing the pre-game and post-game values, an increase of  $AUCE_{drs}$  was observed for the majority of participants (7 of 11 players at PEEP 5 and 5 of 11 players at PEEP 10). Of the seven at PEEP 5 that observed an increase, five were forwards and two were backs, while at PEEP 10, three of the five were forwards and two were backs. After global GPS and game video analysis (Table 6.1), a trend is evident between the number of impacts (scrum, tackle, hit-up, maul, ruck) per match and an increase in lung elastance, while no correlation exists between the number of metres run and changes in  $AUCE_{drs}$ .

## 5.4 Discussion

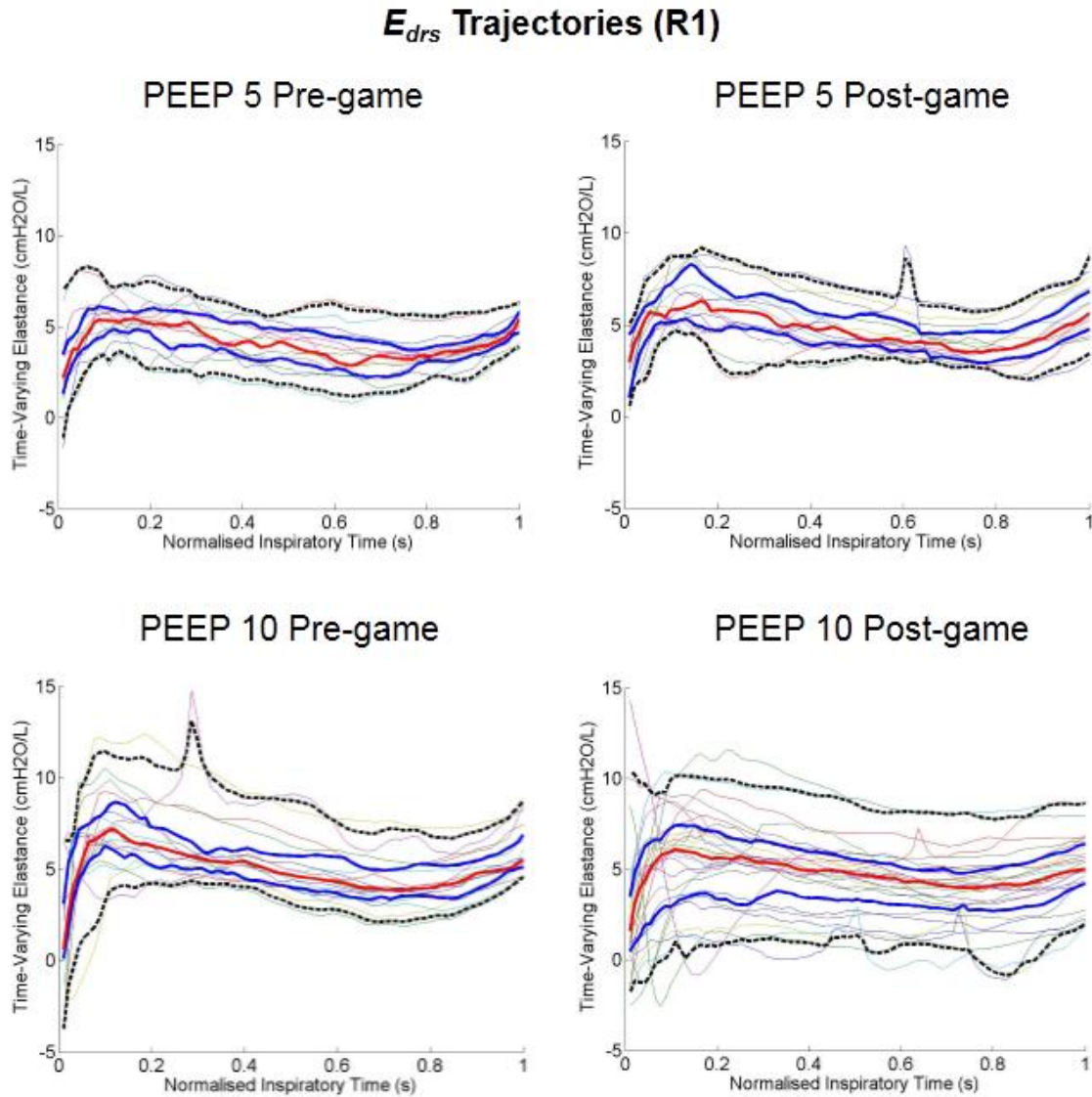
The aims of this study were to examine whether the use of a ventilator and model-based methods could be adapted to assess the respiratory mechanics of a healthy individual, and to test this theory on a group of healthy (no medication) rugby players' following an elite rugby game that could potentially be used as a new means of evaluating "stress" and implementing recovery strategies to maximise performance. This study provided the first proof of a concept of a model-based method to assess the respiratory mechanics of healthy individuals using the  $E_{drs}$  trajectories and  $AUCE_{drs}$ . The  $E_{drs}$  trajectories and  $AUCE_{drs}$  showed that this method adapted from ICU mechanical ventilation can be used to provide insight to respiratory mechanics of healthy (no medication) participants. In addition, the median respiratory elastance measured ( $< 10 \text{ cmH}_2\text{O/L}$ ) placed this cohort in the normal range of healthy individuals suggesting there is no pathological significance to the values observed as a result of a rugby game.

Use of SPONT breathing mode in this trial differs from the synchronized intermittent mandatory ventilation (SIMV) mode previously used with the time varying elastance model. SPONT detects inspiratory effort by a patient and acts to support this, while SIMV ventilates a patient independent of any respiratory effort made. Due to this ventilation independent of

respiratory effort, SIMV is most suited to ventilating sedated individuals, and its use on a non-sedated individual can result in extreme discomfort. As such, the decision was made to employ the SPONT breathing mode for this trial. However, the time varying elastance model is built on underlying respiratory physiology that influences the pressure and volume relationships observed thus will not be influenced by changes in the mode of ventilation.

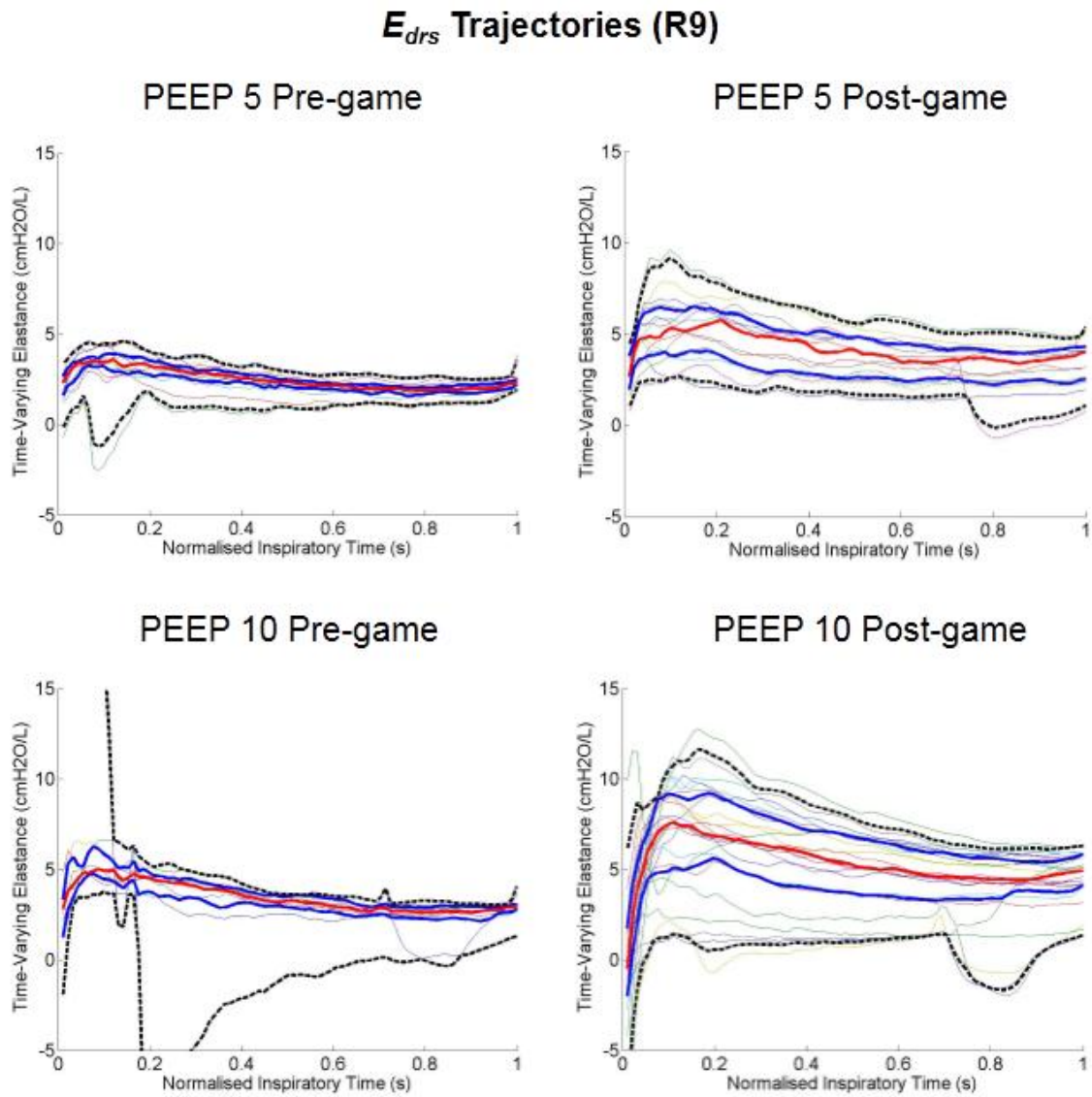
Compared to studies that have focused on mechanically ventilated critically ill patients, it is clear that the respiratory elastance in rugby players before and after a game are within the healthy range (Mason *et al.*, 2010). An increase in respiratory elastance can be interpreted as a higher work of breathing (WOB) (Chiew *et al.*, 2011). The WOB is proportional to the  $AUCE_{drs}$ , where more work is required to fill a given lung with higher  $AUCE_{drs}$  (Chiew *et al.*, 2011). However, absolute changes are relatively small, although percentage changes are larger, and the significance test showed that the  $AUCE_{drs}$  is not significantly different between pre- and post- game over all participants ( $P > 0.05$ ). The relatively small absolute change in  $AUCE_{drs}$  may be due to the small sample size. Equally, there were no catastrophic injuries during the games, and therefore this result was perhaps to be expected.

Critically ill patients with respiratory failure are often seen with respiratory elastance of 25 cmH<sub>2</sub>O/L, or higher (The ARDS Definition Task Force 2012), where healthy human participants have compliant lung elastance values  $< 10$  cmH<sub>2</sub>O/L (Mason *et al.*, 2010). The median  $AUCE_{drs}$  found for this study cohort was  $< 10$  cmH<sub>2</sub>O/L, which corresponds to healthy respiratory elastance. These results suggest that the time-varying elastance model can be used to capture respiratory mechanics of patients who are spontaneously breathing. Equally, the area under the normalised  $E_{drs}$  trajectory  $AUCE_{drs}$  can be used as a surrogate of respiratory elastance (Davidson *et al.*, 2014).



**Figure 5.3.** The time-varying elastance for participant R1 and R9 at PEEP 5 and PEEP 10, in function of a normalized time (1 second), each for pre-game and post-game. No significant changes were found between pre and post-game  $E_{drs}$  trajectory. Each line represents the 5<sup>th</sup>, 25<sup>th</sup>, 50<sup>th</sup>, 75<sup>th</sup> and 95<sup>th</sup> percentile of the  $E_{drs}$ . The 25<sup>th</sup>, 50<sup>th</sup> and 75<sup>th</sup>  $E_{drs}$  are used to calculate the  $AUCE_{drs}$ .





**Figure 5.4.** The time-varying elastance for participant R1 and R9 at PEEP 5 and PEEP 10, in function of a normalized time (1 second), each for pre-game and post-game. No significant changes were found between pre and post-game  $E_{drs}$  trajectory. Each line represents the 5<sup>th</sup>, 25<sup>th</sup>, 50<sup>th</sup>, 75<sup>th</sup> and 95<sup>th</sup> percentile of the  $E_{drs}$ . The 25<sup>th</sup>, 50<sup>th</sup> and 75<sup>th</sup>  $E_{drs}$  are used to calculate the  $AUCE_{drs}$ .

The results suggest that there may be a change in respiratory mechanics after a rugby game. The trend observed between number of impacts and respiratory elastance increase would suggest the impact from the physical nature of the game, rather than respiratory stress due to metres run, may be a greater cause of the observed increase. The significance of such a finding can potentially lead to the development of a new method to determine exercise and impact stress. In combination with other biological markers and testing procedures, an improved understanding of the stresses imposed on athletes, not only in rugby, can be determined and managed correctly to improve the management of player recovery and longevity (Smith 2011). Similar to blood analysis of immune system activation as a guide of infection, this non-invasive and cost-effective type of technology may provide a means of understanding lung damage/disease from exercise and infection that may warrant further investigation from more advanced and invasive procedures. With the normal healthy range considered  $<10 \text{ cmH}_2\text{O/L}$ , an athlete or patient whose elastance exceeds this limit may be considered “at risk” and therefore could be advised to seek advanced medical advice/screening. It may also provide an option as a monitoring tool for progression of lung disease and inflammation both following exercise induced damage and recovery and treatment effects in patients.

### *Conclusion*

In this cohort we did not identify significant changes in player respiratory elastance as a result of a rugby game. However, this study is the first proof of a concept of a model-based method to assess the respiratory mechanics of healthy individuals using the  $E_{drs}$  trajectories and  $AUCE_{drs}$ . The  $E_{drs}$  trajectories and  $AUCE_{drs}$  showed that this method adapted from ICU mechanical ventilation can be used to provide insight to respiratory mechanics of healthy participants that can be used as a more precise measure of lung inflammation/injury that avoids invasive procedures.

### *Limitations and Future Work*

One of the study limitations was the model-based estimated  $AUCE_{drs}$  is unique and not validated with other respiratory function markers. The estimated  $AUCE_{drs}$  of the rugby players are within the healthy range compared to previous studies. However, the use of  $AUCE_{drs}$  as markers for respiratory elastance warrants further investigation for its validity. In particular, pulmonary function tests such as spirometry, body plethysmograph (DuBois *et al.*,

1956; Ghio *et al.*, 1990; Roberts *et al.*, 1991) or other chest imagining techniques (Bodenstein *et al.*, 2009; Hayashi *et al.*, 2013; Heilman 2006; Schmidt 2012) should be carried out in parallel to compare the respective findings.

Another limitation of the study is the time delay that occurred during post-game data collection. The post-game data collection was carried out 17 hours after the rugby game. If there are changes in respiratory mechanics of the players after the game, this delay period potentially allows the player to recover and thus, the changes are minimal and not significant. It is recommended that, in future, the investigation should be carried out immediately after a game (1 or 2 hour time frame) to overcome this limitation. However, as a potential tool for monitoring player recovery and fatigue, it can only be assumed that with elastance values within the healthy range 17 hours post-game, each player may have potentially “recovered”. This information could therefore imply elite amateur rugby union is not of an intensity that causes irreversible lung damage which prevents players from resuming normal training. Comparison between different forms of rugby union could be assessed in conjunction with varying time course measurements.

Future work needs to consider the physical nature of the type of exercise tested. In the case of this cohort, the number of impacts or distance may be related to the increase in elastance as shown in Figure 5.3. The median of  $AUCE_{drs}$  for participants who covered more distance or were involved in a greater number of impacts were generally higher than other participants. However, this study is severely limited by sample size and thus, there is no statistical significance that can be drawn. Follow up studies should incorporate different types of exercise from short, high intensity exercise such as sprinting exercises, to ultra-endurance sports such as multi-sport events. This should identify the cause of the elastance increase.

Future work also needs to consider the ability of elastance measurement in the quantification of lung infection/disease and its ability to monitor progression. Studies following patients before and after treatment from infection/disease may benefit from such technology to understand the time course changes in response to different treatments and their effectiveness.

*What Is Already Known On This Topic*

- Rugby is a high intensity contact sport involving high force impacts. One of the major body parts at risk of contact injury is the trunk.
- In the case of the critically ill, patient-specific respiratory mechanics are assessed to aid clinical decision making.
- Model-based methods are increasingly popular as they provide a unique insight of a patient's condition and response to treatment.

*What this study adds*

- This is the first study to conceptualize the assessment of respiratory mechanics in healthy athletes as a means to monitor post-exercise stress and manage recovery.
- This study is the first to show a model-based method that can assess the respiratory mechanics following exercise using the  $E_{drs}$  trajectories and  $AUCE_{drs}$ .
- It provides a unique insight as to the changes in overall respiratory mechanics.
- This technology may provide useful means of assessing athletes or patients with severe lung inflammation who may require further medical examination.



# Study 3

Involves a clinical investigation of professional rugby players from the Canterbury Rugby Football Union conveyed in one succinct chapter. Selected biomarkers are measured before and after three games in the 2013 ITM Cup to determine their efficacy in measuring changes in acute and chronic physiological stress.

# Chapter 6

## **Assessing the Effectiveness of Selected Biomarkers in the Acute and Cumulative Physiological Stress Response in Professional Rugby Union Through Non-Invasive Assessment**

### **Abstract**

Rugby union is a sport involving high force and frequency impacts making the likelihood of injury a significant risk. The aim of this study was to measure and report the individual and group acute and cumulative physiological stress response during three professional rugby games through non-invasive sampling. Twenty-four professional rugby players volunteered for the study. Urine and saliva samples were collected pre and post three matches. Myoglobin, salivary immunoglobulin A, cortisol, neopterin and total neopterin (neopterin + 7,8-dihydroneopterin) were analysed by high performance liquid chromatography or enzyme linked immunosorbent assay. Significant increases in cortisol, myoglobin, neopterin and total neopterin when urine volume was corrected with specific gravity were observed ( $p < 0.05$ ). Significant decreases in salivary immunoglobulin A concentration were observed for games one and two while secretion rate decreased after games two and three. Significant decreases were seen with the percent of 7,8-dihydroneopterin being converted to neopterin following games two and three. The intensity of three professional rugby games was sufficient to elicit significant changes in the physiological markers selected for our study. Furthermore, results suggest the selected markers not only provide a means for analysing the stress encountered during a single game of rugby but also highlight the unique pattern of response for each individual player.

### **6.1 Introduction**

Rugby union is a high force collision sport played in 100 countries across five continents since its emergence in 1891 (Quarrie and Hopkins 2007). Since the advent of the professional era when more time has been devoted to physical preparation, skill development and tactical aspects of the game, rugby has become faster and more physically demanding with players adapting to the pressures by increasing in size, strength and speed across all positions (Fuller *et al.*, 2012).

Measurement of the stress associated with the game is imperative to gain a greater understanding of the response of a player/team in one of the most physically demanding field sports (Mashiko *et al.*, 2004). Traditionally, these have been assessed through game analysis (McLean 1992; Roberts *et al.*, 2008), injury analysis (Brooks *et al.*, 2005), questionnaire analysis of player fatigue (Cresswell and Eklund 2005) and minimally through biochemical analysis in both amateur and professional rugby matches (Cunniffe *et al.*, 2010; Smart *et al.*, 2008; Takarada 2003). While these studies provide some useful information, they offer a limited and often subjective assessment. They are also typically analysed as a singular event or singular biochemical marker that neglects the effect of cumulative stress and the overall collective stress response of the exercise in question. Cunniffe *et al.* (2011) offered useful information pertaining to the longitudinal effect of professional rugby which highlighted the intensity changes during a season, however this type of analysis is rarely conducted in rugby related sports.

Most studies typically use an invasive procedure which can provide more unwanted stress to the subjects. Participation, accuracy and stress-free collection are three aspects of exercise evaluation required to perform analyses on a regular basis. Tissues most commonly used for physiological examination of exercise include serum, plasma, urine and saliva. While saliva and urine have been used extensively to predict immune system function and psychophysiological stress (Fredericks *et al.*, 2012; Neary *et al.*, 1994), rugby and closely related sports have been commonly assessed through plasma and serum concentrations of myoglobin (Takarada 2003), creatine kinase (McLellan *et al.*, 2010), interleukin-6 (IL-6) and lymphocyte populations (Cunniffe *et al.*, 2010). This does provide valuable information, however the stress and preparative time associated with such procedures provides more hassle and potential loss of participation. Non-invasive sampling protocols that utilize urine and saliva collection are stress and hassle free for participants and require very little prep for analysis.

The measurement of muscle damage, oxidative stress, inflammation, psychophysiological stress and immune system function are all individually capable of providing valuable analysis of an athlete's response to exercise. The combination of these systems however, may provide a more extensive and well-rounded understanding of the overall psychophysiological response in rugby union. Myoglobin, neopterin (NP), cortisol and salivary immunoglobulin



A (sIgA), all of which have been used extensively to assess exercise activities and sports including rugby (Jacks *et al.*, 2002; Nieman *et al.*, 2002; Suzuki *et al.*, 1999; Takarada 2003), may not only provide information about the post-exercise stress response, but could identify player's entering a game, series or season in a fatigued or over-trained state (Cunniffe *et al.*, 2011). The advantage of this analysis allows the individualized assessment and monitoring on a level that is not currently reported in the literature. The presentation of group analysis is commonly conducted in both a sporting and clinical context that often masks the individual response. This is an extremely important aspect in rugby union whose stress is often controlled by the number of impacts a player experiences which is not even across the field (Smart *et al.*, 2008).

The aim of this research is to measure and report the individual and group acute and cumulative psychophysiological stress response during three professional rugby games through non-invasive sampling, and test the effectiveness of the selected five markers in their overall ability to provide an effective assessment of rugby induced stress. It is thought the three games will cause significant and variable changes in all markers on a very individualistic basis that can be explained by the impacts associated with the game.

## 6.2 Methods

### *Subjects*

Twenty-four semi-professional rugby players from a New Zealand provincial team volunteered for the study (Table 6.1.). Three home games were analysed with ~7 days between matches. Of the 24 subjects, 22 were selected to compete in each game, and samples were obtained pre and post-game from 18 of the 22 for game one, 17 for game two and 18 for game three. The experimental protocol was approved by the University of Canterbury Human Ethics Committee, Christchurch, New Zealand and all subjects were informed (Appendix K) of the risks involved in the study before their written consent (Appendix J) was obtained.

*Experimental protocol*

Urine and saliva samples were collected pre-game (approx. 120mins) and immediately post-game (game one:  $37.3 \pm 10.1$ mins, game two:  $41.6 \pm 13.8$ mins, game three:  $33.9 \pm 16.4$ mins) during three selected home games of the 2013 ITM Cup. Immediate post-game sample collection was chosen based on all markers peaking within an hour after exercise (Cunniffe *et al.*, 2010; Mikkelsen and Toft 2005; Schobersberger *et al.*, 2006). Fluids and food were provided by the team (750 mL H<sub>2</sub>O, 375 mL coconut water, 200ml protein shake, fruit, chicken sushi, soup and fried chicken) and each player had been asked to do a 10min cold bath post-game (10 – 12 °C with submergence up to the neck).

*Saliva and urine collection*

Refer to Materials and Methods (Chapter 2.2)

*Game Statistics, Sample Preparation and Analysis*

Refer to Materials and Methods (Chapter 2.2)

**Table 6.1.** Subject characteristics. Data is mean  $\pm$  SD except for experience which is mean + range.

Age (years)	Height (m)	Weight (kg)	Experience (games)
$24.2 \pm 2.9$	$1.87 \pm 0.06$	$103.3 \pm 11.6$	24 (0 - 65)

*Statistical Analysis*

The effect of game variables (number of impacts and number of games played) on the change in concentrations of each marker was tested in a linear mixed effects model fitted with restricted maximum likelihood, conducted in the lme4 package (Bates *et al.*, 2014) in R version 3.1.1 (Team 2014). P values for coefficients of fixed effects were calculated using Satterthwaite's method of denominator synthesis, conducted in the lmerTest package (Kuznetsova *et al.*, 2013) for R. Each marker was analysed as the response variable in a separate model. The fixed predictors in each model were time (pre vs. post-game), the number of impacts in the game sustained by the player (which was replicated at the level of

games per player), and the total number of games played by the player (each player was a replicate). Player identity and game number were included as crossed random effects to account for the non-independence of marker measures from each player, and the potential for some games to have different effects on all players to others. Although there is no true replication of games or players (multiple measures are pseudo replicates), so individual levels of these factors cannot be compared, we tested whether there was significant overall variation explained by the random effects using a likelihood ratio test. Correlations between markers and impacts were calculated using Spearman's rank using the percentage changes from pre to post-game, while two-tailed paired sample t-tests were conducted for the separate analysis of each game for each marker.

### 6.3 Results

Results indicate the intensity of three professional rugby games was sufficient to elicit significant changes in all four biochemical markers selected for this study. Global positioning system data for each game is presented in Table 6.2. Game time, total distance run, player-load and number of impacts were similar across each of the games. The data for each of the biochemical analyses (Fig. 6.1-8) reveal group and individual differences in stress that remain undetected using traditional performance analysis techniques.

**Table 6.2.** GPS game statistics, n=10 (mean  $\pm$  SD).

	<b>Game Time (min)</b>	<b>Distance covered (m)</b>	<b>Player load</b>	<b>No. of impacts</b>
<b>Game 1</b>	72.1 $\pm$ 15.4	5191 $\pm$ 1280	544.7 $\pm$ 87.4	25 $\pm$ 18
<b>Game 2</b>	66.6 $\pm$ 17.0	5368 $\pm$ 1518	565.2 $\pm$ 189.2	22 $\pm$ 12
<b>Game 3</b>	74.4 $\pm$ 18.4	5346 $\pm$ 1509	585.2 $\pm$ 125.2	26 $\pm$ 14

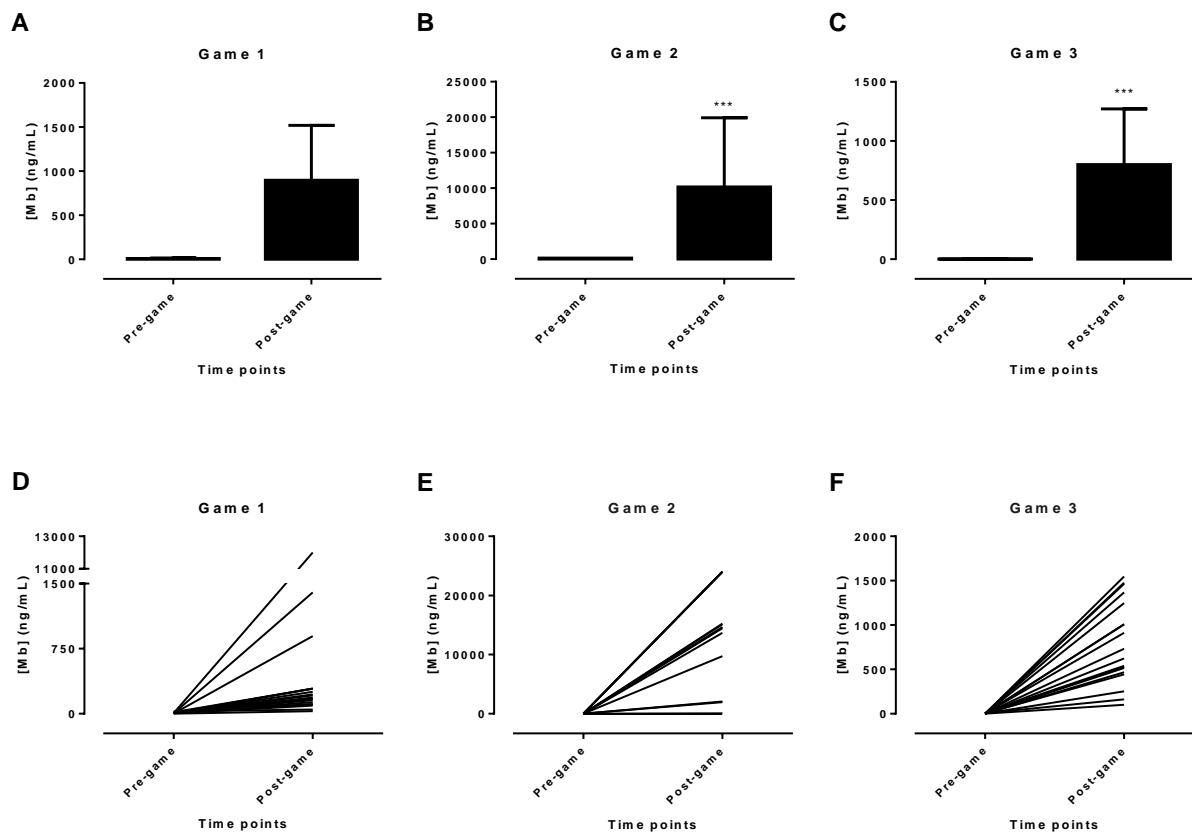
The correlations calculated using Spearman's rank correlation are presented in Table 6.3. There were several positive correlations to be seen following a compiling of the data from all

three games. Expected relationships were observed between the inflammatory markers of NP/SG and total NP/SG, NP/SG and number of impacts, number of impacts and myoglobin, myoglobin and cortisol, and sIgA concentration and sIgA secretions rate. Strong positive trends were also observed between total NP/SG and number of impacts ( $p = 0.057$ ), number of impacts with player-load ( $p = 0.063$ ) and cortisol with sIgA concentration ( $p = 0.066$ ). Unexpected positive relationships were identified between player-load and sIgA secretion rate and cortisol with sIgA secretion rate.

**Table 6.3.** Correlations between variables. Correlations are presented as p and cc (correlation coefficient) values with  $p < 0.05$  representing a significance.  $N = 53$  unless otherwise stated. Bold indicates a statistical significance.  $^+$  denotes a positive correlation,  $-$  denotes a negative correlation.

	Player Load (n=25)	No. of impacts	Myoglobin	Cortisol	sIgA secretion rate	[sIgA]	NP (SG)
No. of impacts	0.063	-	-	-	-	-	-
Myoglobin	0.716	<b>0.028<sup>+</sup></b>	-	-	-	-	-
	-	(cc=0.50)	-	-	-	-	-
Cortisol	0.166	0.380	<b>0.047<sup>+</sup></b>	-	-	-	-
	-	-	(cc=0.38)	-	-	-	-
sIgA secretion rate	<b>0.038<sup>+</sup></b>	0.097	0.576	<b>0.011<sup>+</sup></b>	-	-	-
	(cc=0.39)	-	-	(cc=0.31)	-	-	-
[sIgA]	0.146	0.617	0.270	0.066	<b>0.001<sup>+</sup></b>	-	-
	-	-	-	-	(cc=0.71)	-	-
NP (SG)	0.858	<b>0.046<sup>+</sup></b>	0.217	0.491	0.246	0.734	-
	-	(cc=0.57)	-	-	-	-	-
Total NP (SG)	0.334	0.057	0.719	0.324	0.675	0.788	<b>0.000<sup>+</sup></b>
	-	-	-	-	-	-	(cc=0.81)

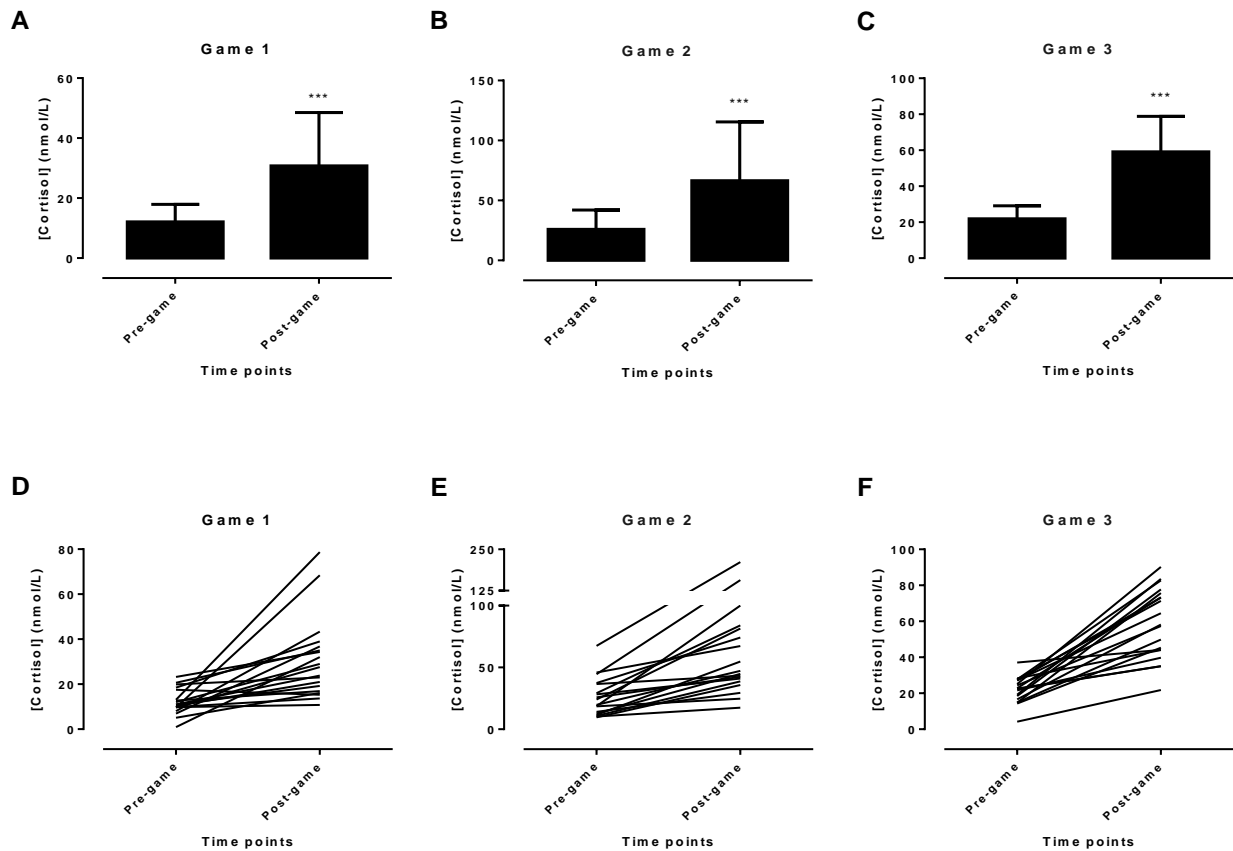
Group urinary myoglobin post-game concentrations increased above pre-game concentrations for all three games (Fig 6.1A-C), but only significantly for games two ( $p < 0.001$ ) and three ( $p < 0.001$ ) which is largely due to the large standard deviation. There was a 91, 1832 and 336 fold increase in group mean concentrations following games one, two and three respectively. All pre-game levels started at approximately the same starting point ( $< 5$  ng/mL) while post-game concentrations varied greatly player to player resulting in large error bars and large differences in the degree of muscle damage experienced (Fig. 6.1D-F).



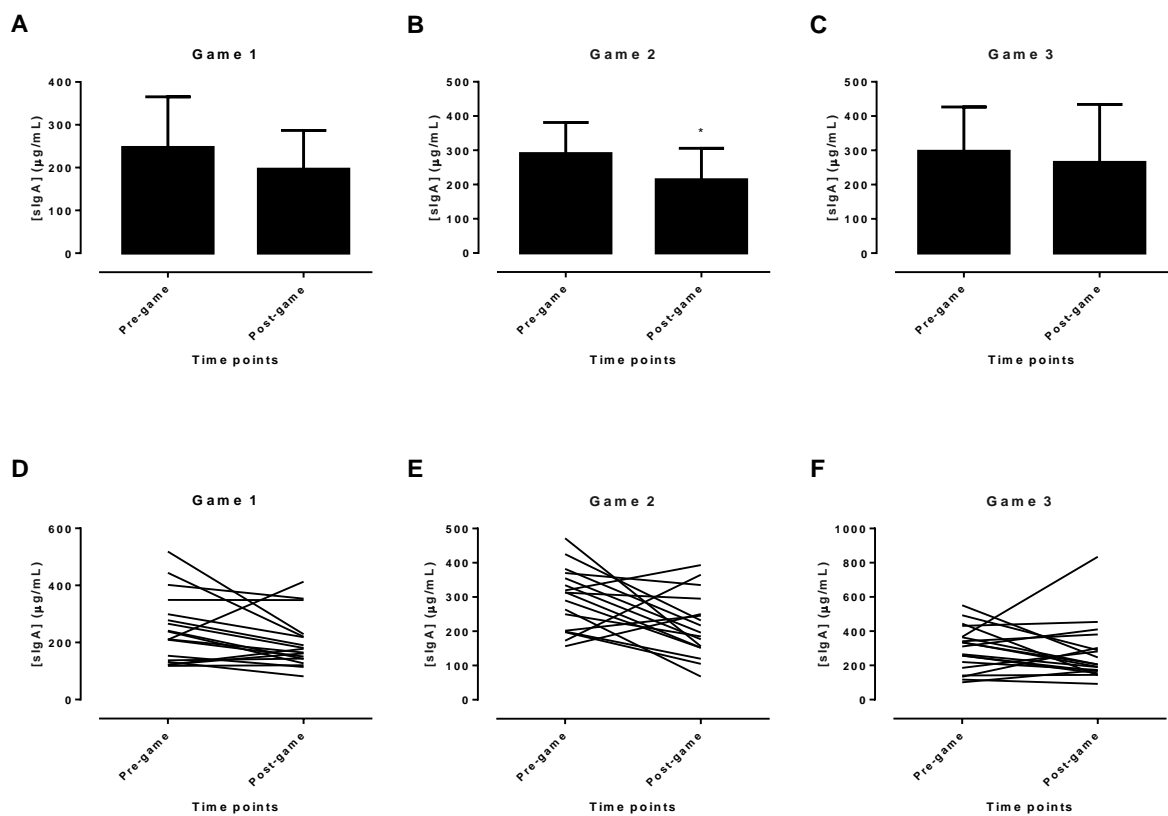
**Figure 6.1.** Urinary myoglobin concentrations following three semi-professional rugby games. Group data is represented by A, B and C, while individual myoglobin concentrations are represented by D, E and F. Data is mean  $\pm$  SD. (\*\*\*)  $p < 0.001$ .

Salivary cortisol concentrations increased by 2.54, 2.57 and 2.73 fold following each game for games one, two and three respectively ( $p < 0.001$ ) (Fig. 6.2A-C). Post-game concentrations could be predicted by the pre-game concentrations ( $p < 0.001$ ) while there were significant differences observed between the games for pre and post-game concentration ( $p < 0.001$ ) and between the individual subjects ( $p < 0.001$ ) (Fig. 6.2D-F). There was also sufficient evidence to suggest the more impacts a player experiences, the less their post-game cortisol concentration would increase ( $p < 0.001$ ), while the more games a subject participates in, the lower their post-game salivary cortisol concentration ( $p < 0.001$ ). However, when subjects experience more impacts and compete in more games, post-game concentrations do not decrease to the same degree as the separate effects ( $p < 0.001$ ).

Salivary immunoglobulin A concentration was seen to significantly decrease for game two only ( $p = 0.019$ ) (Fig. 6.3A-C). There was a 1.26, 1.38 and 1.12 fold decrease in group mean concentrations following games one, two and three respectively. There was also sufficient evidence to suggest impacts had a significant effect on the post-game concentration ( $p = 0.026$ ), while the more games a player participated in did not affect sIgA concentration. Analysed separately however, certain subjects showed a decreasing trend over time (Fig. 6.8A). Furthermore, significant differences were observed between the subject's post-game concentration ( $p < 0.001$ ) (Fig. 6.3D-F) that was able to be explained by the pre-game concentration ( $p = 0.033$ ), while no evidence was found to suggest the number of games played affected post-game concentrations. Similarly, sIgA secretion rate decreased significantly for game two only ( $p = 0.002$ ) (Fig. 6.4A-C). There was also sufficient evidence to suggest the post-game secretion rate could be explained by the pre-game secretion rate ( $p < 0.001$ ), number of impacts significantly affected post-game secretion rate ( $p = 0.015$ ), number of games played had no effect on pre to post-change, and the games and subjects were all significantly different from one another ( $p < 0.001$ ) (Fig. 6.4D-F).

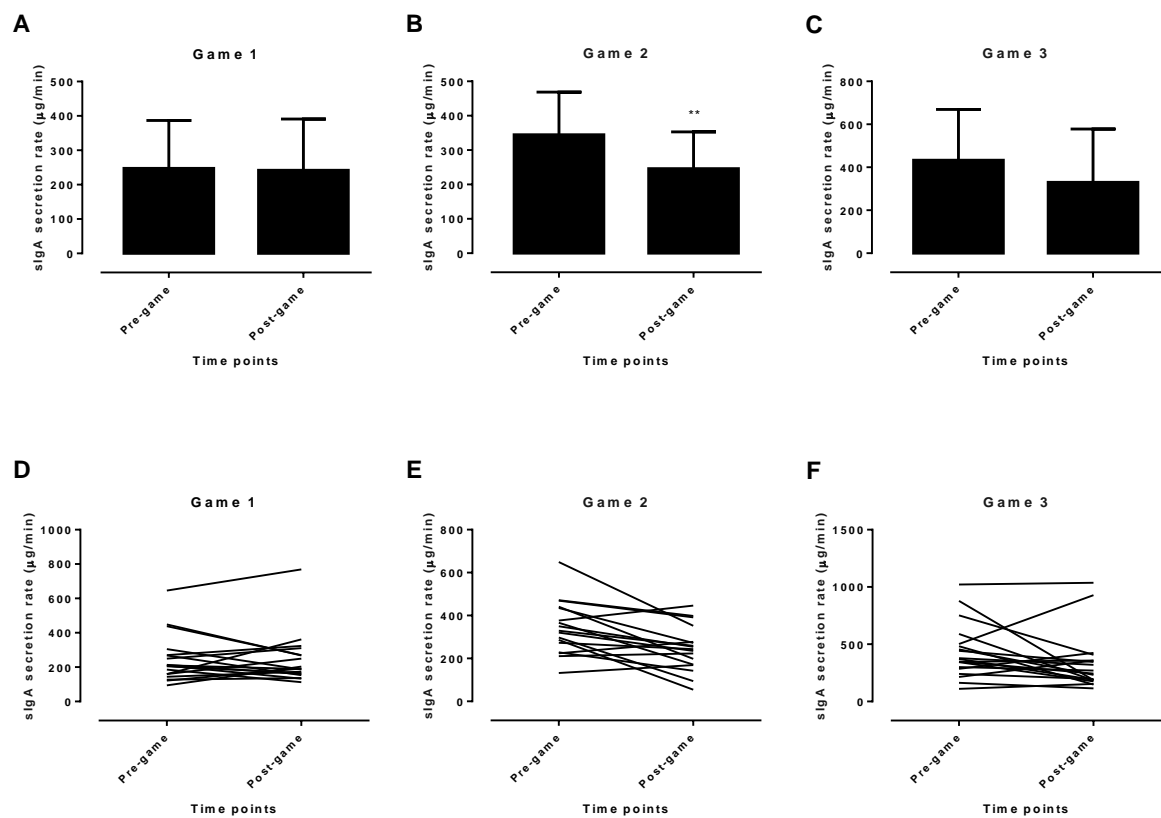


**Figure 6.2.** Salivary cortisol concentrations following three semi-professional rugby games. Group data is represented by A, B and C, while individual myoglobin concentrations are represented by D, E and F. Data is mean  $\pm$  SD. (\*\*\*)  $p < 0.001$ .



**Figure 6.3.** sIgA concentrations following three semi-professional rugby games. Group data is represented by A, B and C, while individual myoglobin concentrations are represented by D, E and F. Data is mean  $\pm$  SD. (\*  $p < 0.05$ ).

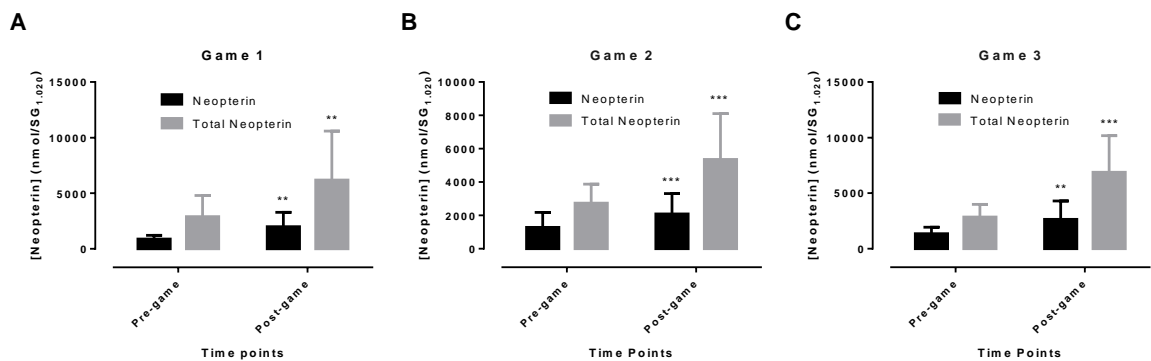




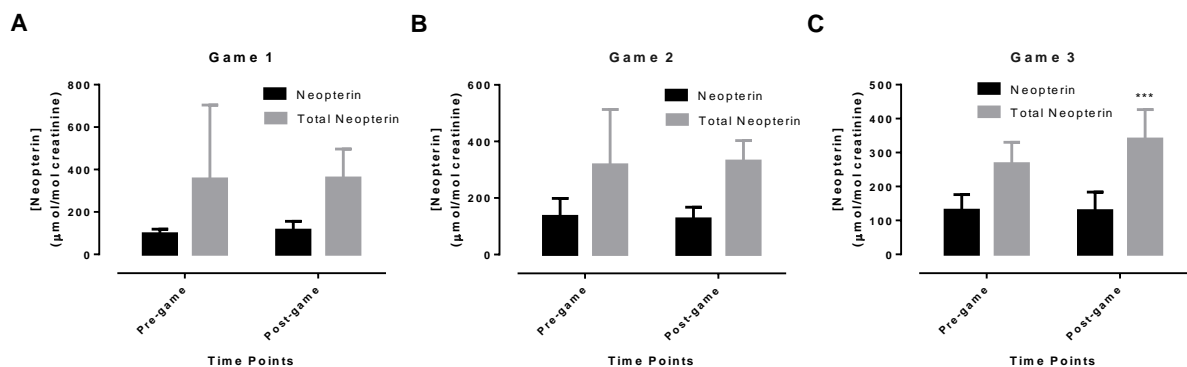
**Figure 6.4.** sIgA secretion rate following three semi-professional rugby games. Group data is represented by A, B and C, while individual myoglobin concentrations are represented by D, E and F. Data is mean ± SD. (\*\* p < 0.01).

Levels of immune system activation and inflammation following all three games are presented in Figures 6.5 and 6.6. Urinary NP corrected with SG increased significantly for games one ( $p = 0.002$ ), two ( $p = < 0.001$ ) and three ( $p = 0.001$ ), while post-game total NP concentrations increased 2.14, 1.96, and 2.41 fold above pre-game concentrations for game one ( $p = 0.003$ ), two ( $p = < 0.001$ ) and three ( $p = < 0.001$ ) respectively. There is sufficient evidence for an effect of impacts on NP/SG ( $p = 0.02$ ) and minor effect on total NP/SG ( $p = 0.086$ ), while both NP/SG ( $p = 0.005$ ) and total NP/SG ( $p = 0.05$ ) post-game concentrations were significantly decreased when a subject participated in more games. When analysed separately however, certain subjects showed an increasing trend in concentration when participating in all three games for NP (Fig. 6.8E) and total NP (Fig. 6.8F). Furthermore, there were significant differences ( $p < 0.01$ ) observed between the players for post-game NP/SG and total NP/SG. There is also evidence showing there was a significant difference between the games for both NP and total NP, while post-game concentrations could be predicted by the pre-game concentrations of both markers ( $p < 0.001$ ). When urine volume is corrected with creatinine however (Fig. 6.6), significant change is only observed for total NP following game three ( $p < 0.001$ ). Similar to SG correction, there is sufficient evidence for an effect of impacts on post-game concentration for NP and total NP, while there were significant differences between the post-game concentrations for both markers between games and significant differences between subjects also ( $p < 0.001$ ).

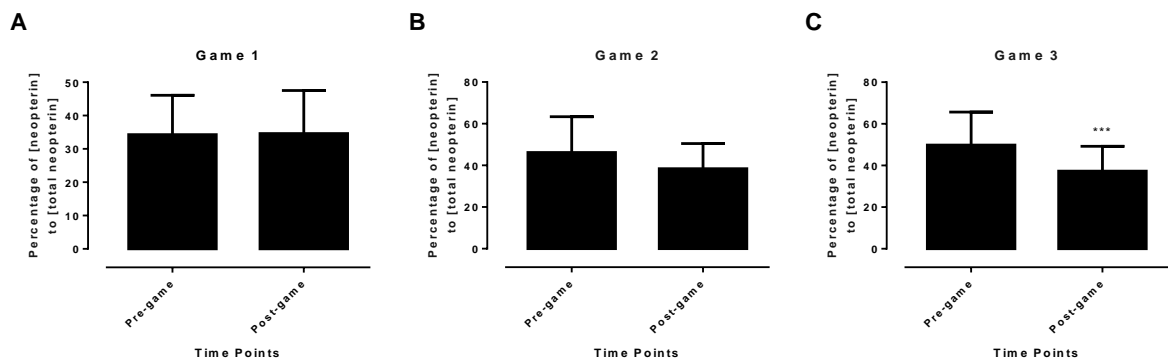
Figure 6.7 represents the percentage of NP to total NP when urine volume was corrected with SG. While games one and two showed no change, game three showed a significant decrease ( $p = < 0.001$ ) in the amount of 7,8-dihydroneopterin being converted to NP. Again there was a similar pattern of individual variation (data not shown).



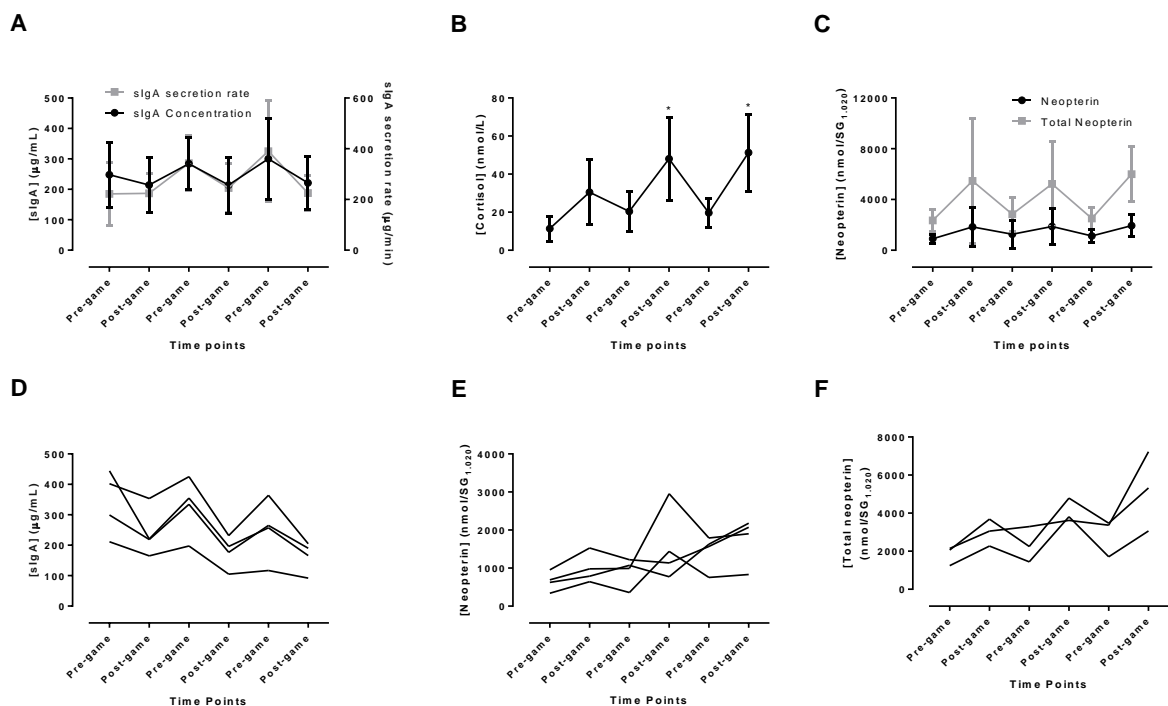
**Figure 6.5.** Urinary NP and total NP corrected with SG following three semi-professional rugby games. Data is mean  $\pm$  SD. (\*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ )



**Figure 6.6.** Urinary NP and total NP corrected with creatinine following three semi-professional rugby games. Data is mean  $\pm$  SD. (\*\*\*  $p < 0.001$ ).



**Figure 6.7.** Percentage of NP to total NP corrected with SG following three semi-professional rugby games. Data is mean  $\pm$  SD. (\*\*\*)  $p < 0.001$ .



**Figure 6.8.** Group data of the subjects who played in all three games ( $n = 11$ ) for sIgA concentration and secretion rate (A), cortisol (B) and NP and total NP (C). Selected subjects over the course of the three games that showed a decrease in sIgA concentration (D), increase in urinary NP (E) and total NP (F) concentrations when corrected for SG. Data is mean  $\pm$  SD. (\* $p < 0.05$ ).

## 7.4 Discussion

Professional rugby union is a severe intensity sport that causes changes in selective biomarkers in response to the intermittent and high force impact nature of the game. The level of competition in this study caused significant elevations in muscle damage which is in accordance with similar research (Smart *et al.*, 2008). The detection of intracellular muscle proteins represents ultra-structural damage to the muscle cell membrane as a result of the blunt force trauma commonly associated with the game and similar to that previously noted (Smart *et al.*, 2008). Additionally, it causes activation of inflammatory processes and associated oxidative stress that are so often associated with sport and exercise of a high intensity (Margeli *et al.*, 2005; McLellan *et al.*, 2011a; Viguie *et al.*, 1993). In conjunction with these structural changes, elevations in psychophysiological stress and suppression of the immune system were observed. These have been previously noted in other impact sports (Hoffman *et al.*, 2005) that can lead to an increased vulnerability to clinical and subclinical infection (Cunniffe *et al.*, 2010; Nieman 1996). The compiled acute stress assessment incorporating all five markers may provide a greater understanding of the intensity of professional rugby union and why it is considered the most physically demanding field sport in the world (Mashiko *et al.*, 2004).

Moreover, professional rugby union seems to cause an accumulation of stress, more commonly referred to as fatigue or over-reaching/over-training. This has been shown to occur previously in rugby union (Finaud *et al.*, 2006b; Maso *et al.*, 2004) and suggests the combination of game stress and associated training may cause under-recovery leading to detrimental effects on performance (Budgett 1998). With questionnaires about perceived fatigue and performance so commonly utilized in the assessment of individual players (Flynn *et al.*, 1994), the utilization of biochemical analysis may open up a more precise and factual understanding of the term in impact related sports.

Quite often the analysis of the individual player or patient (in clinical terms) is neglected or unpublished. Due to variable genetic and physiological profiles of individuals that can be attributed to age, gender and ethnicity, responses to various stimuli can be significantly different. Previously shown in body-builders during a week of training (Lindsay *et al.*, 2014a) and recently in elite amateur rugby players (Lindsay *et al.*, 2014b), the response to exercise stress can vary greatly between athletes. This research reinforces that already

presented in rugby union with large inter-individual variations in all selected markers (Fig. 6.1 – 4). While some show significant levels of muscle damage, stress and immune system suppression suggesting they experienced a higher number of impacts, covered a greater distance during the game, were on debut or unusually nervous, or simply more prone to psychophysiological stress than others, other more fortunate players do not. This may be attributed to confidence (Morgan 2011), adaptation/experience, or lack of game time. Either way, it is evidently clear the use of biochemical analysis has a place in professional rugby union for the individual assessment of psychophysiological stress. Its ability to differentiate between the “responders” and “non-responders” is essential for management and recovery of each player as a separate entity.

A major part of this research was to investigate the effectiveness of selected markers to identify acute and cumulative changes of stress in professional rugby union. Limited research is available on changes in biochemical markers following contact related sport (Cormack *et al.*, 2008; Hoffman *et al.*, 2005; Johnston *et al.*, 2013), let alone rugby union (Lindsay *et al.*, 2014b). Selected for their specificity in clinical and exercise based research, each of the five markers presented significant changes in their response to the stress. Urinary myoglobin to our knowledge has only been used once to assess muscle damage in rugby union (Lindsay *et al.*, 2014b), while its identification in plasma following rugby union was shown to correlate with number of impacts (Takarada 2003). Differences in concentrations between the studies may be attributed to the level of competition, with the previous study investigating elite amateur rugby, or the time to analysis with the current study completing analysis immediately after collection. It does however, provide a direct quantification of the muscle damage experienced, and due to its extremely fast elimination kinetics and time to peak (Suzuki *et al.*, 1999), presents itself as a worthy replacement of muscle damage quantification both clinically and in a sporting context. The variability from game to game however, potentially due to impacts, highlights both the relative instability of myoglobin in urine and explains the large individual variation among subjects and between games (Fig. 6.1D-F). Recommendations include pH adjustment, immediate analysis and avoidance of multiple freeze-thaw cycles to maintain stability (Alterman *et al.*, 2007; Chen-Levy *et al.*, 2005; Gabow *et al.*, 1982; King *et al.*, 2010; Naka *et al.*, 2005; Wu *et al.*, 1994)..

Likewise, salivary cortisol and IgA both significantly changed which is in accordance with previous research and likely a result of the high force impacts (Lindsay *et al.*, 2014b; Nieman *et al.*, 2002). Both compounds effectively represent acute and cumulative group and individual psychophysiological stress and immune system function respectively. The significant elevation in cortisol and IgA suppression provides sufficient evidence that they can be an effective psychophysiological stress and immune system marker in rugby union, but which may also have the ability to measure individual stress adaptation over time and over-training (Gleeson *et al.*, 2000; Maso *et al.*, 2004). The significant decline in IgA in game two may also suggest it was of a greater intensity than the other two games. The non-invasive assessment and variable increase from pre to post-game among individual subjects makes them simple, stress free and sensitive in their predictions of stress. Methodology limitations do exist however. Measured in  $\text{nmol/L}^{-1}$ , creams such as hydrocortisone and/or blood that contains even higher concentrations than cream can quite often enrich a cortisol sample leading to a false high value (Marini and Cabassi 2002). Careful collection and monitoring of external cortisol containing creams can alleviate this problem. Meanwhile, known correlations with lymphocyte populations and sIgA concentration suggests sIgA may be a good predictor of overall immune system status. Cunniffe *et al.* (2010) have shown similar decreases in circulating concentrations of T-lymphocytes and natural killer cells immediately following a rugby game, further illustrating the ability of IgA to act as an effective marker of immune system function.

Similarly, the immediate rise in total NP is associated with game-related impacts and is in accordance with other studies examining inflammation in rugby (Cunniffe *et al.*, 2010) and rugby league (Mendham *et al.*, 2012). Meanwhile, the significant decline in post-game urinary total NP following the participation of two or more games is potentially indicative of an adaptation process. The increasing trend observed for certain individuals however, (Fig. 6.8B and C) provides evidence of potentially impaired recovery, and why individual assessment is so imperative. This justifies the use of total NP as a detection method for players in a “fatigued” state, and similar to that previously reported in body-builders (Lindsay *et al.*, 2014a). Consequently, total NP could become a marker of choice for measuring inflammation in sport due to its direct relation to immune system activation through interferon- $\gamma$  (IFN- $\gamma$ ) stimulation and communication between activated T-cells and macrophages (Wachter 1992; Wachter *et al.*, 1989). Furthermore, the immediate post-game

rise in urinary total NP provides an added advantage over traditional inflammatory markers such as tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), C-reactive protein (CRP) and creatine kinase (CK) that are measured in serum and peak 14 - 38 hours post exercise (Cunniffe *et al.*, 2010; Ispirlidis *et al.*, 2008; Ostrowski *et al.*, 1999). The immediate quantification of an acute phase inflammatory response without delay is an important aspect for player management and similar to the response of IL-6 (Pedersen *et al.*, 2003; Suzuki *et al.*, 2003).

Commonly thought to be a result of the large aerobic component of the game and a research area that is relatively unexplored, the increase in NP (oxidative stress) seems to be influenced by the number of impacts a player experiences and indicative of rugby union as a sport that increases oxidative stress. Finaud *et al.* (2006b) showed intense periods of rugby competition and training induce significant increases in markers of oxidative stress which can be implicated with the arrival of overtraining. Coupled with a decrease in post-game concentration following multiple games, urinary NP may therefore offer a simplistic and new approach for player fatigue management.

Furthermore, the quantification of psychophysiological stress was able to be calculated in a non-invasive manner. The stress-free, hassle-free and non-disruptive collection of samples is essential for subject participation on a large scale where technology of this nature could be utilized in team sports. Muscle damage for example is routinely measured through serum and plasma changes in creatine kinase, troponin, carbonic anhydrase and others (Kyröläinen *et al.*, 1998; McLellan *et al.*, 2010; Sorichter *et al.*, 1997) which requires lengthy prep time and a stressful situation for the subject. Through sensitive enzyme linked immunosorbent assay (ELISA), the current methodology has shown to be capable of identifying post-exercise muscle damage providing samples are analysed immediately. Additionally, immune system function through saliva analysis is equally efficient at gauging immune health as a result of its close correlation with other known parameters (lymphocytes). The stress and hassle free collection and simple analysis by ELISA and HPLC avoids unnecessary analysis and invasive procedures that have so often been employed to measure exercise stress (Lancaster *et al.*, 2004; Nielsen *et al.*, 1996).

This research has also provided added information regarding urine volume normalization in sports testing. The significant increase in NP and total NP post-game using SG correction



only may be a result of several factors. While both are good predictors of hydration status (Miller *et al.*, 2004), rugby significantly increases urinary creatinine post-game (data not shown). This is in accordance with previous exercise studies which may prevent any meaningful observation with a urinary analyte (Anderson *et al.*, 1982; Calles-Escandon *et al.*, 1984; Decombaz *et al.*, 1979). The rise in SG (data not shown) may provide evidence of dehydration status and metabolic waste production, yet it is not overwhelmed and still allows for correct measurement. It would seem logical to correct urine volume using SG following exercise which is the current practice by the World Anti-Doping Agency (WADA), however in any other circumstance, either option provides the necessary correction. Careful consideration has to be given to samples with a SG < 1.003 and > 1.030 as they are considered over-hydrated and dehydrated respectively, and should not be used for urine volume correction (Alessio *et al.*, 1985; Cone *et al.*, 2009).

This research has several applications. The values observed can be used in the future as reference values and a bench mark against severe intensity sport. In addition, the non-invasive methodology and reliability of the biochemical markers of this study are stress and hassle free allowing for higher rates of participation and simple analysis; providing sample collection and handling is carried out correctly and efficiently. The ability to individually assess a player by this technique is especially important in monitoring and gauging the health of a player that has potential recovery and training implications. Combined reporting of group analyses and individual tests on a single protocol or game needs to be considered as the intra- and inter variation among subjects and between games/protocols may be drastically different.

### *Conclusion*

Professional rugby union is a high intensity sport that induces significant changes in markers of psychophysiological stress. A lack of correlation between some of the markers and game analysis suggest that each marker in our study measured a unique component of overall player stress. The non-invasive nature of urine and saliva collection makes this type of analysis player-friendly and repeatable in a professional context. Results indicate these markers offer a greater potential depth of individualised data for coaches, over and above that possible through traditional performance analysis techniques such as Global positioning system (GPS) tracking.



# Study 4

Comprises a clinical investigation of professional rugby player's as part of a Super 15 franchise during the 2014 Super 15 competition. The investigation contains five specifically designed chapters measuring psychological and physiological stress through non-invasive biochemical analysis and examines the relationship between their acute and chronic changes in association with currently utilized player work-load technologies.

# Chapter 7

## Impact Induced Muscle Damage and Urinary Pterins in Professional Rugby: 7,8-Dihydroneopterin Oxidation by Myoglobin.

### Abstract

Muscle damage caused through impacts in rugby union is known to increase oxidative stress and inflammation. Pterins have been used clinically as markers of oxidative stress, inflammation and neurotransmitter synthesis. This study investigates the release of myoglobin from muscle tissue due to force related impacts and how it is related to the subsequent oxidation of 7,8-dihydroneopterin to specific pterins. Effect of iron and myoglobin on 7,8-dihydroneopterin oxidation were examined *in vitro* via SCX-HPLC analysis of neopterin, xanthopterin, 7,8-dihydroxanthopterin. Urine samples were collected from 25 professional rugby players pre and post four games and analysed for myoglobin by ELISA, and 7,8-dihydroneopterin oxidation products by HPLC. Iron and myoglobin oxidized 7,8-dihydroneopterin to neopterin, xanthopterin and 7,8-dihydroxanthopterin at concentrations at or above 10 $\mu$ M and 50 $\mu$ g/mL respectively. All four games showed significant increases in myoglobin, neopterin, total neopterin, biopterin and total biopterin which correlated between each variable ( $p < 0.05$ ). Myoglobin and iron facilitate 7,8-dihydroneopterin oxidation to neopterin and xanthopterin. *In vivo* delocalisation of myoglobin due to muscle damage may contribute to oxidative stress and inflammation after rugby. Increased concentrations of biopterin and total biopterin may indicate production of nitric oxide and monoamine neurotransmitters in response to the physical stress.

### 7.1 Introduction

Intense exercise is commonly associated with an increase in reactive oxygen species (ROS). Exceeding the anti-oxidant defence capacity can result in oxidative alteration to lipids, DNA and proteins (Roebuck 1999), induce muscle damage (Duarte *et al.*, 1993), and contribute to the effect of delayed onset muscle soreness (Aoi *et al.*, 2004). In a sport that is dominated by high force collisions requiring consistent aerobic exercise interspersed with high speed anaerobic exertions (Cunniffe *et al.*, 2009), rugby union results in significant trauma and the release of intramuscular proteins. However, there is minimal research (Takahashi *et al.*,

2007) quantifying the effect and relationship of rugby related stress on changes in oxidative status.

Heme proteins such as Mb have been postulated as markers of oxidative stress in the urine of rhabdomyolysis patients and shown to correlate strongly with rugby related impacts (Reeder *et al.*, 2002; Takarada 2003). This has been demonstrated as the principal causative factor in rhabdomyolysis-induced renal failure as it undergoes auto-oxidation to the ferric form promoting lipid peroxidation through the  $\text{Fe}^{3+}/\text{Fe}^{4+}$  redox cycle (Giulivi and Cadenas 1998). This cycling has been revealed to elevate urinary isoprostane concentrations in animal models 7.3-fold above controls through its accumulation in the kidney (Moore *et al.*, 1998). Heme initiated oxidative stress is possible when ultra-structural damage to the muscle membrane results in leakage of intracellular proteins (Brown and Hill 1991). In a rugby context, force related impacts are the most likely cause of this damage (Smart *et al.*, 2008) and we speculate whether there is a relationship between the elevation in myoglobin and markers of oxidative stress.

Oxidative stress is typically assessed by changes in serum and urine antioxidant concentration, oxidatively modified molecules, and the cellular redox balance (Finaud *et al.*, 2006a). Derived pterins of 7,8-dihydroneopterin (dihydroneopterin) offer a non-invasive alternative. They are part of the pterin family synthesized by macrophages and monocytes upon interferon- $\gamma$  (IFN- $\gamma$ ) stimulation during inflammation (Giesege *et al.*, 2008a). Neopterin (NP) and 7,8-dihydroneopterin elevation are indicative of an acute or chronic inflammatory response and associated oxidative stress (Giesege *et al.*, 2008a), while xanthopterin (XP), biopterin (BP) and total BP (BP + 7,8-dihydrobiopterin ( $\text{BH}_2$ ) + tetrahydrobiopterin ( $\text{BH}_4$ )) represent changes in oxidative stress and synthesis of essential monoamine neurotransmitters and NO (Daubner *et al.*, 2011; Kwon *et al.*, 1990). Used to diagnose genetic and psychiatric disorders and diseases associated with inflammation and oxidative stress (Hamerlinck 1999), they have also been shown to rise in response to intense exercise (Mizutani *et al.*, 1994).

The conversion of dihydroneopterin to NP *in vivo* has only been demonstrated to occur with via hypochlorite oxidation (Widner *et al.*, 2000). Hypochlorite is generated by neutrophils and to a lesser extent macrophages during inflammation (Halliwell and Gutteridge 1999). Several different ROS have been revealed to oxidize dihydroneopterin to dihydroxathopterin and XP (Giesege *et al.*, 2001a; Laura Dántola *et al.*, 2008), however minimal evidence has

elucidated other potential mechanisms for NP production. Myoglobin’s oxidizing potential and release following rugby related impacts offers a possible alternative for dihydroneopterin oxidation to NP.

The purpose of this study is to identify whether chelated iron and Mb are capable of oxidizing dihydroneopterin to NP, dihydroxanthopterin and XP *in vitro*, and whether this reaction is replicated *in vivo* following the hypothesized Mb release from rugby related impacts. We also compare and contrast the pre and post-game concentrations of NP, dihydroneopterin, BP, total BP, XP, Mb, game related impacts, number of games played, and total distance covered in four professional rugby games to assess the effect of rugby on inflammation, oxidative stress and markers of vasodilation/monoamine neurotransmitter synthesis.

7.2 Methods

Subjects

Thirty-seven professional rugby players volunteered for the study. Of the 37, 23 were selected for each game, and of those 23, 16 provided pre and post-game samples for game one, 17 for game two, 14 for game three and 18 for game four. Table 7.1 shows details of the subjects enrolled in the study. The experimental protocol was approved by the University of Canterbury Human Ethics Committee, Christchurch, New Zealand and all subjects were informed (Appendix M) of the risks involved in the study before their written consent was obtained (Appendix L).

**Table 7.1.** Subject characteristics. Data is mean ± SD.

Age (years)	26.0 ± 3.5
Height (m)	1.86 ± 0.07
Weight (kg)	104.5 ± 9.3

#### *Experimental Protocol and Sample Collection*

Urine samples were collected pre-game (approx. 120 mins) and immediately post-game (within 90 mins) for four games.

#### *Sample collection*

Refer to Material and Methods (Chapter 2.2)

#### *Oxidation by Fe-EDTA and Myoglobin*

Refer to Materials and Methods (Chapter 2.2)

#### *Sample Preparation and Analysis and Video-Analysis*

Refer to Materials and Methods (Chapter 2.2)

#### *Statistical Analysis*

The effect of game variables (number of impacts, number of games played and distance covered) on the change in concentrations of each marker was tested in a linear mixed effects model fitted with restricted maximum likelihood, conducted in the lme4 package (Bates *et al.*, 2014) in R version 3.1.1 (Team 2014). P values for coefficients of fixed effects were calculated using Satterthwaite's method of denominator synthesis, conducted in the lmerTest package (Kuznetsova *et al.*, 2013) for R. Each marker was analysed as the response variable in a separate model. The fixed predictors in each model were time (pre vs. post-game), the number of impacts in the game sustained by the player (which was replicated at the level of games per player), the total number of games played by the player (each player was a replicate) and the distance covered by each player. Player identity and game number were included as crossed random effects to account for the non-independence of marker measures from each player, and the potential for some games to have different effects on all players to others. There is no true replication of games or players (multiple measures are pseudo replicates), so individual levels of these factors could not be compared. We therefore tested whether there was significant overall variation explained by the random effects using a likelihood ratio test.

After completing mixed models analyses, significant correlations were observed between all fixed factors. As such, each fixed factor was analysed separately to ascertain the significance of its effect using a Bonferroni correction. Significance was therefore set at  $p < 0.017$ . Urinary NP/SG, BP/SG, total BP/SG, XP/SG and Mb data were analysed separately per game using a two-tailed paired samples T-test and presented as mean  $\pm$  SEM. Oxidation of dihydroneopterin by Fe-EDTA and Mb were analysed using one way analysis of variance (ANOVA) and presented as mean  $\pm$  SEM, while the relationship between post-game concentrations of urinary Mb and impacts, and the percentage change in NP, total NP, BP, total BP and XP were analysed by Spearman's rank correlation and represent samples from all four games. Percentage changes were chosen to represent the actual physiological effect of the games irrespective of the pre-game concentration which may be affected by multiple variables. All samples were analysed in duplicate and significance was measured as \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ .

### 7.3 Results

The addition of Fe-EDTA at concentrations  $\leq 1 \mu\text{M}$  did not cause oxidation of dihydroneopterin, nor did it increase the concentration of NP, XP or dihydroxanthopterin significantly. Concentrations at or above  $10 \mu\text{M}$  however, caused a steady decrease in dihydroneopterin and an increase in NP, XP and dihydroxanthopterin (Fig. 7.1A). The percentage of dihydroneopterin oxidized to NP was highest at  $10 \mu\text{M}$  (33.9%), followed by a drop at  $100 \mu\text{M}$  (19.9%), which decreases as the concentration of Fe-EDTA increases. This change can be attributed to XP and dihydroxanthopterin which make up 12% and 69% respectively at 5 mM.

The time course oxidation of dihydroneopterin by  $100 \mu\text{M}$  Fe-EDTA significantly increased the NP, XP and dihydroxanthopterin concentration after zero minutes and continued to elevate over the course of the experiment. Simultaneously, dihydroneopterin decreased in concentration, however it is not until 30 minutes that a significant change is observed (Fig. 7.1B). Meanwhile, at the 0 and 15 minute mark, the percentage of dihydroneopterin appearing as NP is as high as 60%, however as the sudden decrease of dihydroneopterin occurs, the percentage significantly decreases consistently over time (Fig. 7.1B) with dihydroxanthopterin making up 76.7% of the lost dihydroneopterin at 240 minutes.

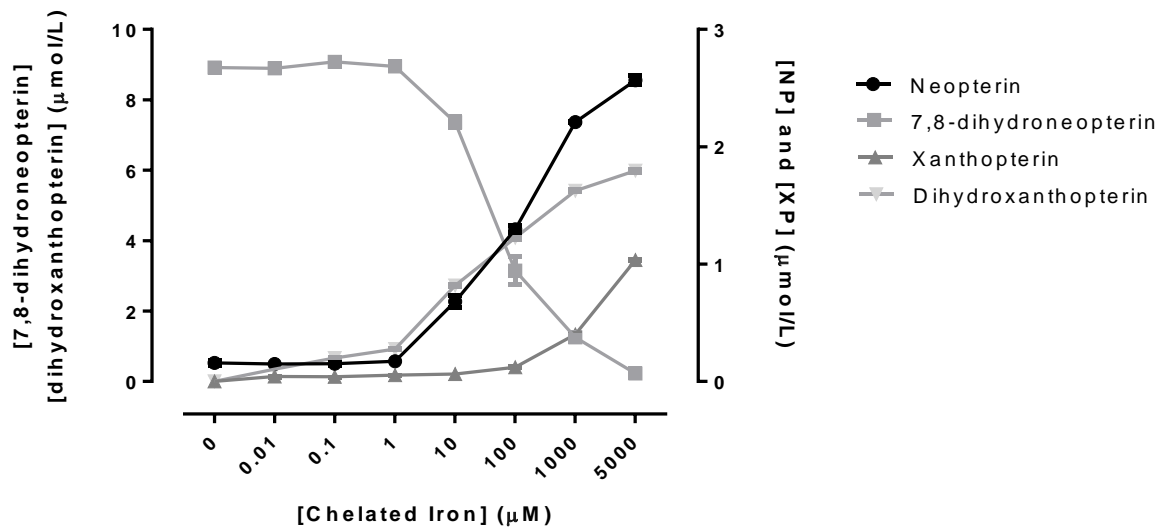


The addition of Mb to 20  $\mu\text{M}$  dihydroneopterin did not cause any significant change in its concentration until levels reached 500  $\mu\text{g/mL}$ , at which point it decreases to 57% of the starting concentration. The rise in NP, XP and dihydroxanthopterin significantly changed with the addition of 5  $\mu\text{g/mL}$  and continued to increase with higher concentrations (Fig. 7.2A). The earlier change in NP correlates with the high observed percentage (74%) of NP appearing as a result of the oxidative loss of dihydroneopterin. This percentage drops dramatically to 12.1% with a 10-fold increase in Mb concentration (Fig. 7.2A) with XP present as 1.59  $\mu\text{M}$  and dihydroxanthopterin as 1.28  $\mu\text{M}$  corresponding to 44.3% and 35.8% respectively of the loss.

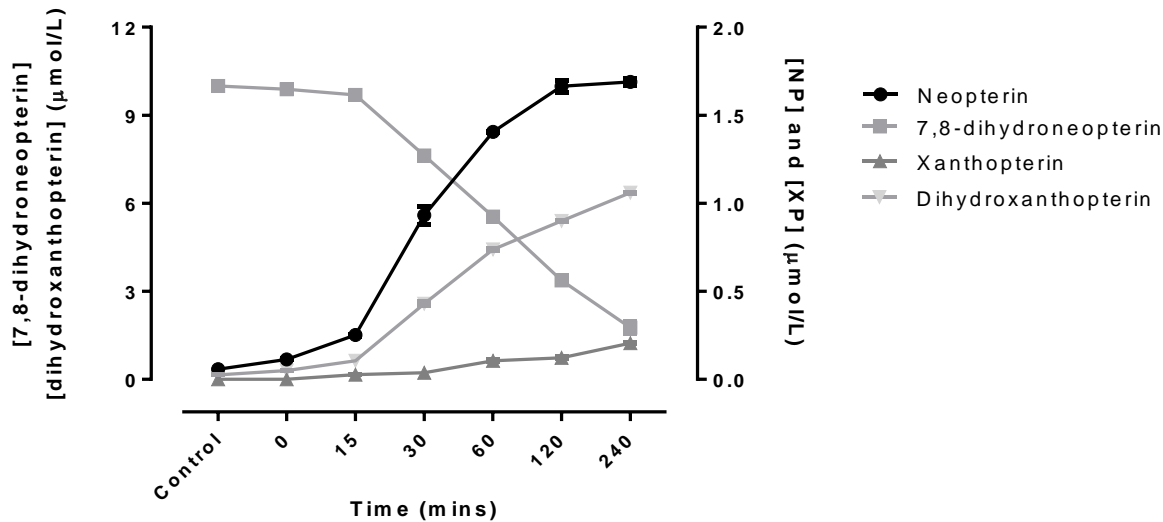
The rate of dihydroneopterin oxidation over time by Mb was similar to Fe-EDTA. The addition of 500  $\mu\text{g/mL}$  Mb caused non-significant changes in dihydroneopterin, NP and XP after 0 minutes and significant changes at 15 minutes for XP and 30 minutes for all compounds thereafter. The decrease in dihydroneopterin is mirrored by the increase in NP, XP and dihydroxanthopterin (Fig. 7.2B), however the percentage of dihydroneopterin lost that is measured as NP is no higher than 14% and is significantly higher at the longer time points (Fig. 7.2B). The remaining dihydroneopterin loss is comprised of 47% XP and 43% dihydroxanthopterin.

Following all four games, urinary myoglobin was significantly increased in games one and four (Fig. 7.3) that was significantly affected by the number of impacts ( $p = 0.004$ ). Urinary NP meanwhile, significantly increased from pre to post-game (2.82, 1.37, 1.47 and 1.67 fold increase respectively) (Fig. 7.4A) with significant differences observed between the post-game concentrations of each game ( $p = 0.003$ ) and between each subject ( $p = 0.011$ ). The change from pre to post-game was shown to be significantly higher if a player covered more distance ( $p = 0.0286$ ) which was further increased when more impacts were experienced ( $p = 0.0735$ ). However, when a subject played more games, the post-game NP concentration did not increase as much above pre-game levels ( $p = 0.035$ ).

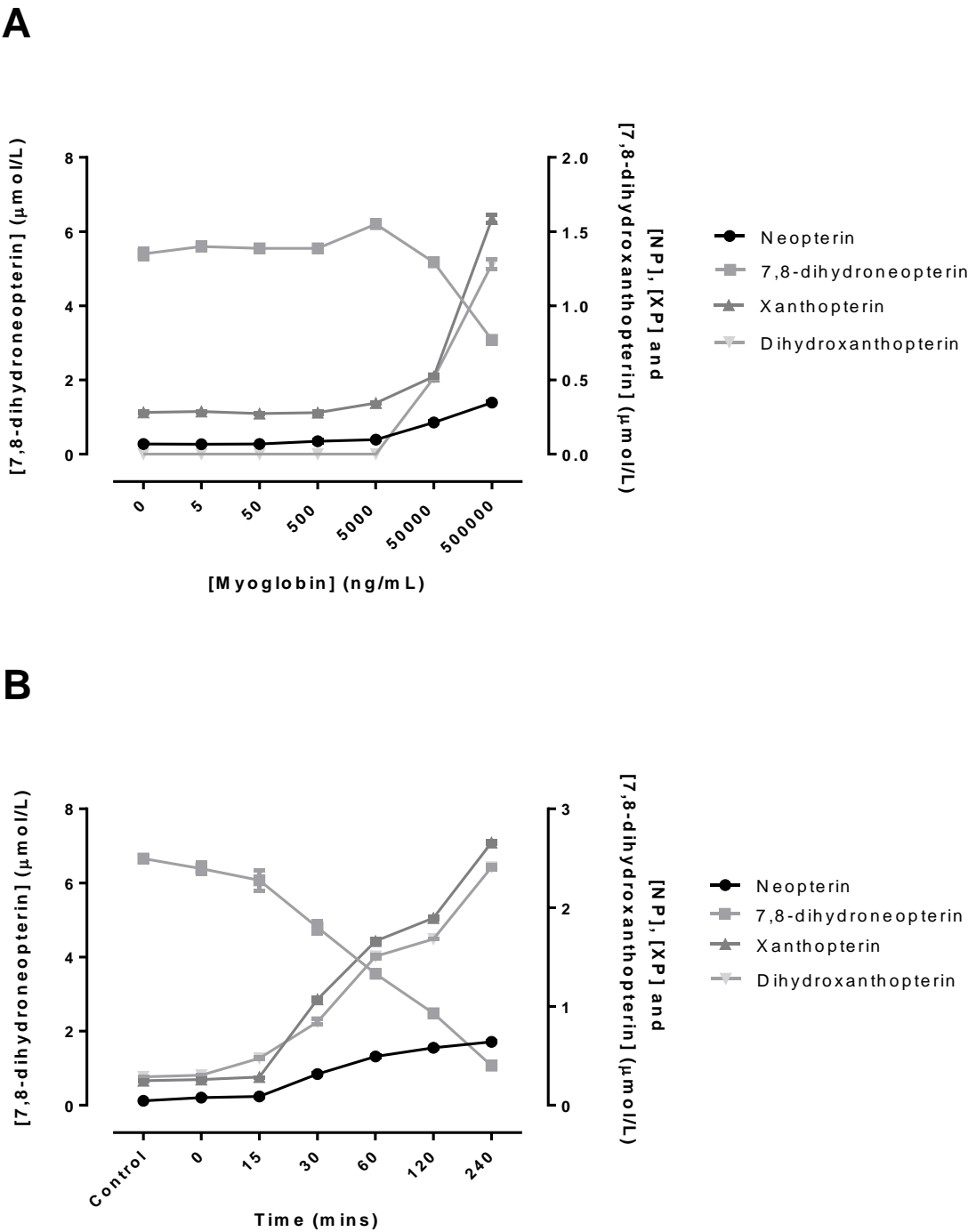
A



B



**Figure 7.1.** Effect of chelated iron on dihydroneopterin oxidation. 20  $\mu\text{M}$  dihydroneopterin was incubated with A) varying concentrations of Fe-EDTA (0 – 5 mM) at 37°C for two hours and B) 100  $\mu\text{M}$  Fe-EDTA at 37°C for 0 - 240mins. Quantification was conducted using SCX-HPLC and data is presented as mean  $\pm$  SEM.



**Figure 7.2.** Effect of human myoglobin on dihydroneopterin oxidation. 20  $\mu$ M dihydroneopterin was incubated with A) varying concentrations of myoglobin (0 – 500  $\mu$ g/mL) at 37°C for two hours and B) 500  $\mu$ g/mL myoglobin at 37°C for 0 - 240mins. Proteins were precipitated with ACN following incubation. Quantification was conducted using strong-cation exchange (SCX)-HPLC and data is presented as mean  $\pm$  SEM.

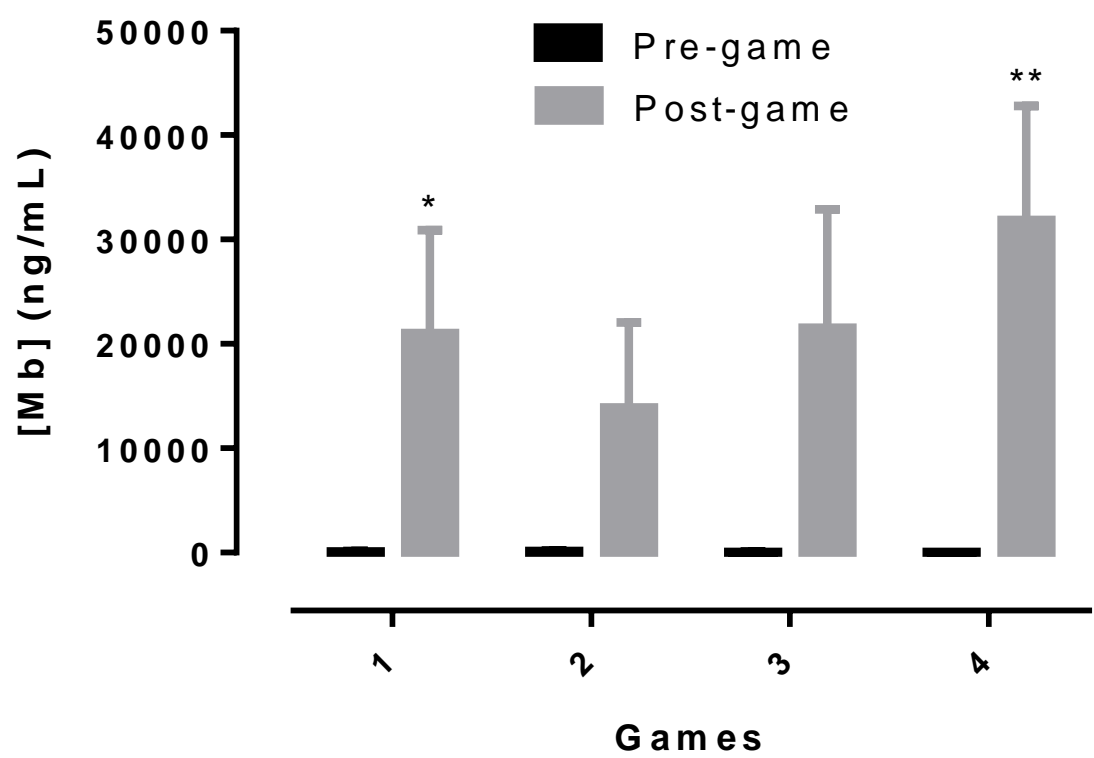
Total urinary NP showed similar observations. Post-game concentration significantly increased above pre-game (1.89, 1.62, 1.45 and 1.56 respectively) (Fig. 7.4B) that was shown to be significantly affected by the pre-game concentration ( $p < 0.001$ ). Unlike NP however, there was no difference between the player response or the game response. When analysed separately, both impacts ( $p = 0.005$ ) and distance covered ( $p = 0.0141$ ) significantly affected the post-game concentration, while the more games played, the less the total NP will increase ( $p = 0.0829$ ).

The change in urinary BP was significantly different between games ( $p < 0.001$ ) which resulted in significant increases in games three and four only (Fig. 7.5A). The 1.36, 1.28, 1.36 and 1.33 fold increase in BP for all four games was slightly affected by the pre-game concentration ( $p = 0.07$ ), while the post-game concentration was significantly increased when a player experienced more impacts ( $p = 0.004$ ) and covered more distance ( $p = 0.016$ ).

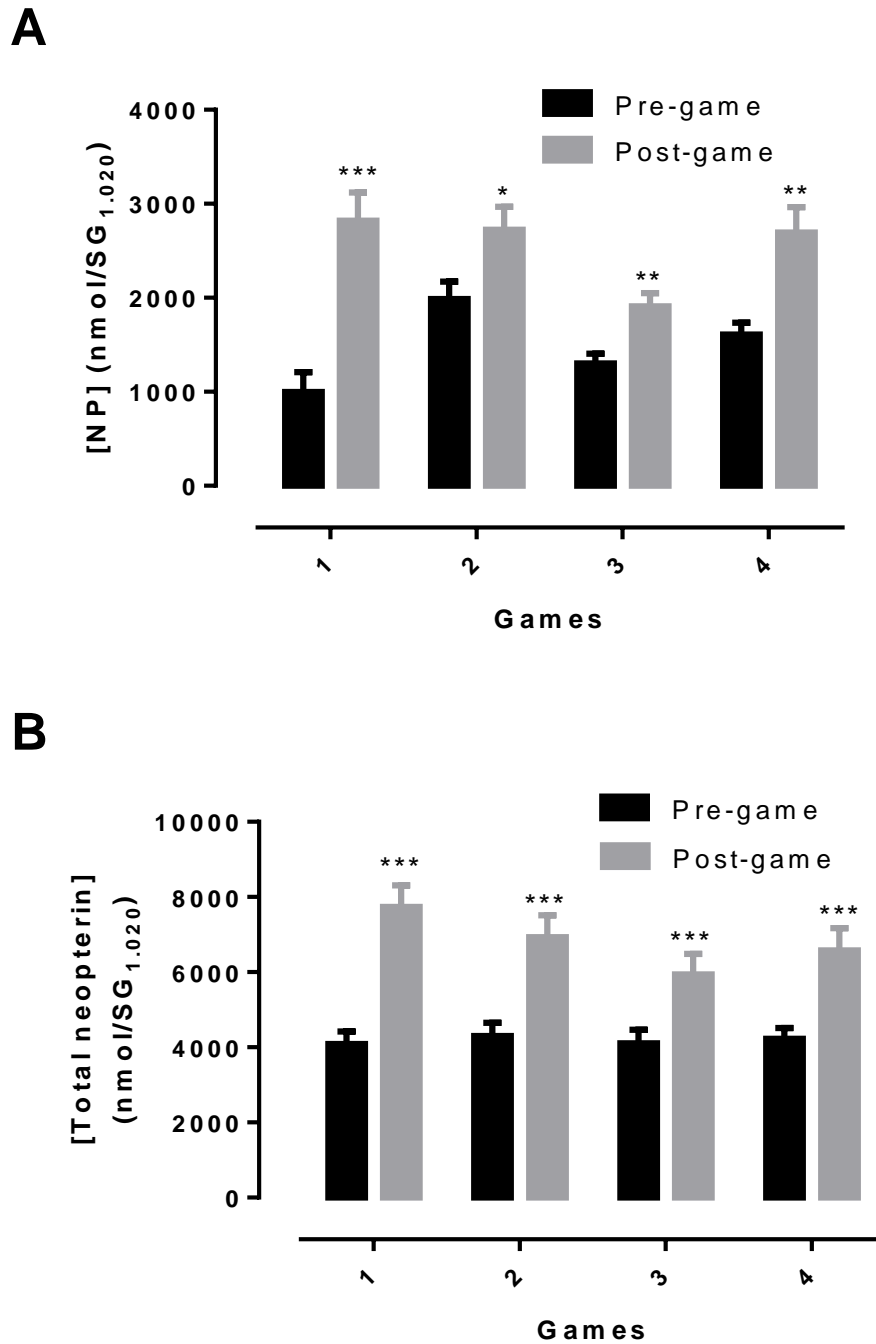
Total BP was shown to increase by 1.57, 1.85, 1.49 and 1.63 fold from pre to post-game for games one to four respectively (Fig. 7.5B). While the post-game concentration could be significantly predicted by the pre-game concentration ( $p < 0.001$ ), it was observed to increase significantly when more impacts were experienced ( $p = 0.001$ ) and more distance covered ( $p = 0.002$ ). However, when more games were played, post-game total BP did not increase as much ( $p = 0.045$ ) above pre-game.

Changes in urinary XP were significantly different between games ( $p < 0.001$ ). A 1.39, 1.57, 1.54 and 0.84 fold change from pre to post-game for games one to four respectively was observed which were affected by the number of impacts experienced ( $p = 0.018$ ) (Fig. 7.6). There was only a significant increase above pre-game levels in game two, while the post-game concentration could be significantly predicted by the pre-game concentration ( $p < 0.001$ ).

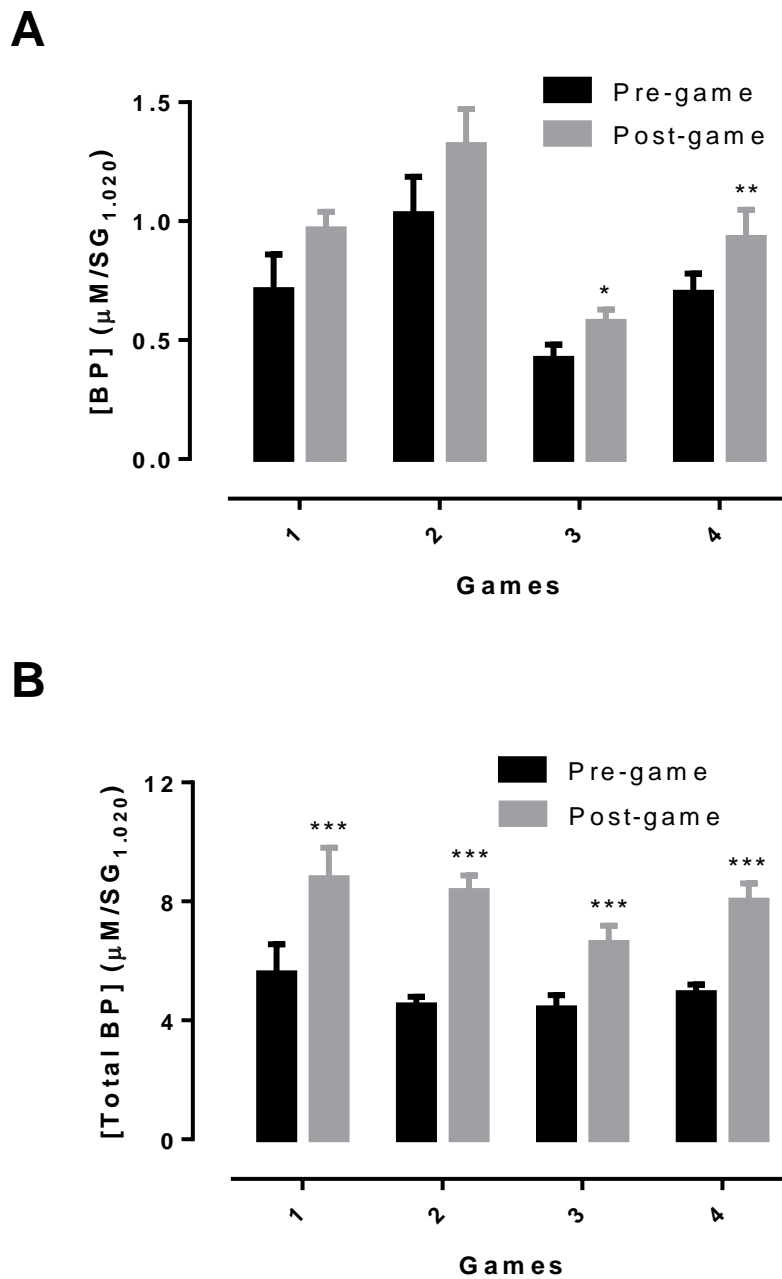
Correlations between percentage changes of the individual measured variables are presented in Table 7.2. Spearman's rank correlation identified the strongest relationship between total NP and total BP ( $cc = 0.814$ ,  $p < 0.001$ ) while strong relationships were also identified between NP with total NP, BP and total BP. Impacts during the games showed a significant correlation with all markers except XP, while Mb was correlated with NP and total NP.



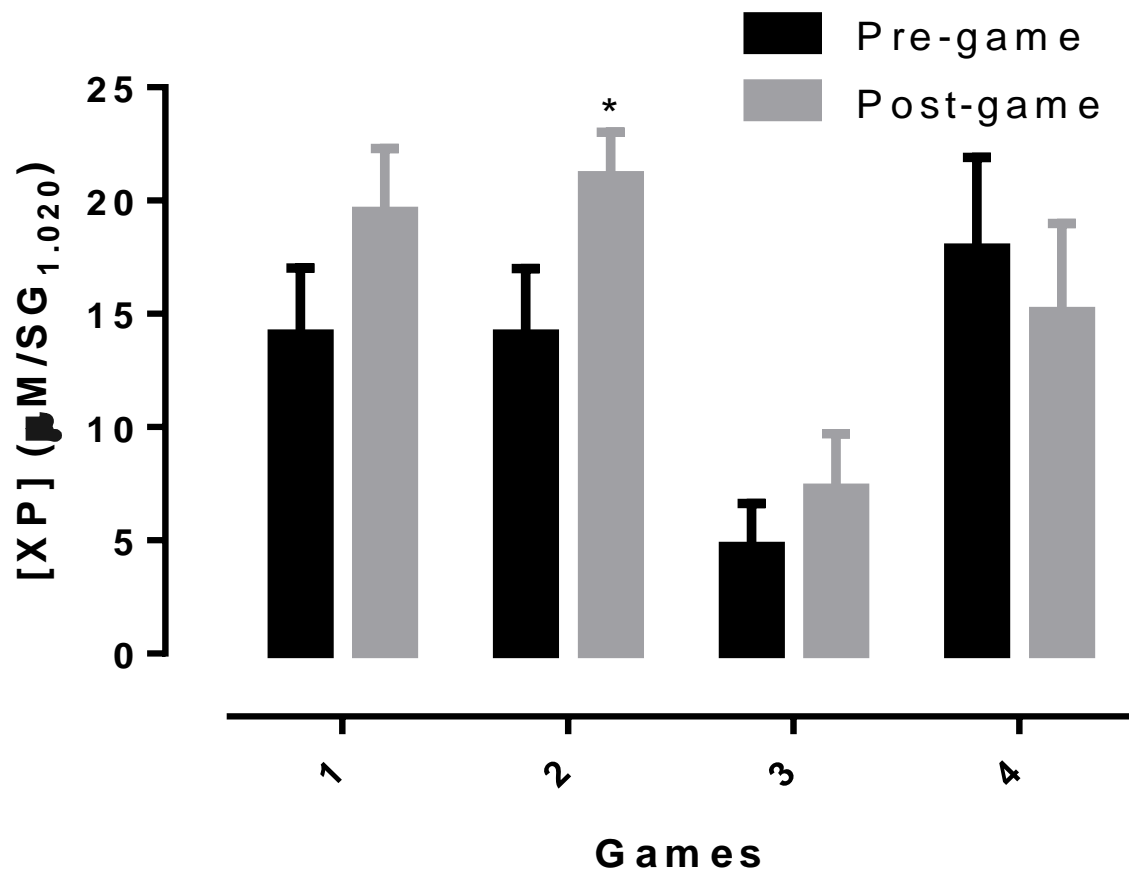
**Figure 7.3.** Mean pre and post-game urinary myoglobin concentration for games one (n = 16), two (n = 17), three (n = 14) and four (n = 18). Data is presented as mean  $\pm$  SEM. (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).



**Figure 7.4.** Mean pre and post-game urinary NP (A) and total NP (B) concentrations for games one (n = 16), two (n = 17), three (n = 14) and four (n = 18). Data is presented as mean  $\pm$  SEM. (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).



**Figure 7.5.** Mean pre and post-game urinary BP (A) and total BP (B) concentrations in games one ( $n = 16$ ), two ( $n = 17$ ), three ( $n = 14$ ) and four ( $n = 18$ ). Data is presented as mean  $\pm$  SEM. (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).



**Figure 7.6.** Mean pre and post-game urinary XP concentrations for games one ( $n = 16$ ), two ( $n = 17$ ), three ( $n = 14$ ) and four ( $n = 18$ ). Data is presented as mean  $\pm$  SEM. (\*  $p < 0.05$ )



**Table 7.2.** Correlations using the individual subject data between Mb, impacts and pre to post-game percentage changes in NP, total NP, XP, BP and total BP. Correlations are presented as p values and correlation coefficients (cc) with  $p < 0.05$  representing a significance and highlighted in bold. (N = 65).

		Impacts	NP	Total NP	XP	BP	Total BP
<b>Mb</b>	p	<b>0.002</b>	<b>0.002</b>	<b>0.013</b>	0.294	0.101	0.055
	cc	<b>0.386</b>	<b>0.380</b>	<b>0.305</b>	0.188	0.205	0.239
<b>Impacts</b>	p	-	<b>&lt;0.001</b>	<b>0.001</b>	0.399	<b>0.015</b>	<b>0.005</b>
	cc	-	<b>0.481</b>	<b>0.409</b>	0.142	<b>0.300</b>	<b>0.346</b>
<b>NP</b>	p	-	-	<b>&lt;0.001</b>	0.341	<b>&lt;0.001</b>	<b>&lt;0.001</b>
	cc	-	-	<b>0.733</b>	0.014	<b>0.752</b>	<b>0.663</b>
<b>Total NP</b>	p	-	-	-	0.738	<b>&lt;0.001</b>	<b>&lt;0.001</b>
	cc	-	-	-	0.060	<b>0.453</b>	<b>0.814</b>
<b>XP</b>	p	-	-	-	-	0.266	0.420
	cc	-	-	-	-	0.237	0.145
<b>BP</b>	p	-	-	-	-	-	<b>0.008</b>
	cc	-	-	-	-	-	<b>0.526</b>

## 7.4 Discussion

Fe and Mb are capable of oxidizing dihydroneopterin to NP, dihydroxanthopterin and XP *in vitro*. Significant elevation in all selected markers during at least one of the four games demonstrates rugby union induces measureable changes in selected pterins and markers of muscle damage (Fig. 7.3 - 6), while the correlation between impacts and all markers except XP, points to a partial relationship (Fig. 7.3 - 6). Furthermore, the positive correlations between some of the markers illustrates the connection between several physiological and biochemical systems stimulated by professional rugby including muscle damage (Mb), innate immune activation (total NP), oxidative stress (NP, XP, BP) and compounds responsible for vasodilation and monoamine neurotransmitter synthesis (total BP) (Fig. 7.7). Although correlations do not exist between all markers, subjects who experienced significant elevations

in muscle damage, all experienced high amounts of oxidative stress as measured by the oxidation products NP, BP and XP. Muscle damage however, is not a prerequisite for oxidative stress, but simply contributes to the overall effect.

Fe-EDTA is able to oxidize dihydroneopterin at concentrations at or above 10  $\mu\text{M}$  (Fig. 7.1A) which is similar to iron's known pivotal role in the oxidation of saturated fatty acids (Minotti 1993). In our studies 7,8-dihydroneopterin is primarily oxidized to dihydroxanthopterin (69%) through Ferrous/Feric iron ( $\text{Fe}^{2+}/\text{Fe}^{3+}$ ) cycling which is in accordance with previous findings (Laura Dántola *et al.*, 2008). The remaining loss is principally composed of NP, XP, other polar substances and non-pterinic compounds (Laura Dántola *et al.*, 2008) due to the stability of NP and XP (data not shown) (Widner *et al.*, 2000). Furthermore, the immediate change in NP (Fig. 7.1B) indicates instantaneous dihydroneopterin oxidation (Fig. 7.1A) that is similar to the immediate lipid peroxidation of brain synaptosomes by  $\text{Fe}^{2+}$  (Braugher *et al.*, 1986). Similarly, Mb concentrations that mimic those found in the post-game urine of rugby players (50 – 500  $\mu\text{g/mL}$ ) were shown to oxidize dihydroneopterin (Fig. 7.2). The reaction occurs through the tyrosine peroxy radical of the iron containing porphyrin ring which has been shown to initiate Mb induced lipid peroxidation (Fig. 7.2A) (Giulivi and Cadenas 1998), while time course changes suggest total oxidative stress during a game may depend on the timing of muscle injury.

The impacts commonly associated with rugby union are the most likely cause for the increase in Mb (Table. 7.2) which has been previously noted (Takarada 2003). Loss of muscle cell membrane integrity can result in Mb delocalization causing renal damage due to isoprostane elevation (Moore *et al.*, 1998). Furthermore, the immediate rise in NP indicates Mb's potential ability to instantly contribute to oxidative stress which is further corroborated by the correlation between Mb and NP identifying impacts as a primary source of muscle damage induced oxidative stress. It also provides new relative information regarding the mechanism responsible for NP production, which has only been shown to occur through the reaction with hypochlorite *in vivo* (Widner *et al.*, 2000). Hypochlorite is released from activated neutrophils and macrophages (Halliwell and Gutteridge 1999) during periods of inflammation which suggests much of the NP produced comes from these sites. However, this evidence suggests that at least some part of the measured NP in urine and plasma may also be present as a result of trauma induced muscle damage which provides alternative mechanisms for the

immediate post-exercise rise in NP following muscle damaging exercise (Lindsay *et al.*, 2014b).

Significantly greater oxidative damage may arise from the formation of heme-protein cross link species (Mb-H) following oxidation of Mb. This reaction has been shown to enhance low-density lipoprotein (LDL) and NADH oxidation several fold compared to native Mb (Vuletic *et al.*, 2000). The significant elevation in post-game BP indicates oxidant formation and the likelihood of Mb-H production and subsequently increased oxidative stress (Reeder *et al.*, 2002). In contrast, Mb's instability may cause loss of its oxidative potential due to factors such as temperature, pH, unidentified urinary compounds smaller than 10kDa, and time to analysis (Chen-Levy *et al.*, 2005), while its extremely fast elimination kinetics (Suzuki *et al.*, 1999) may also contribute to its inability to act as an oxidizing agent.

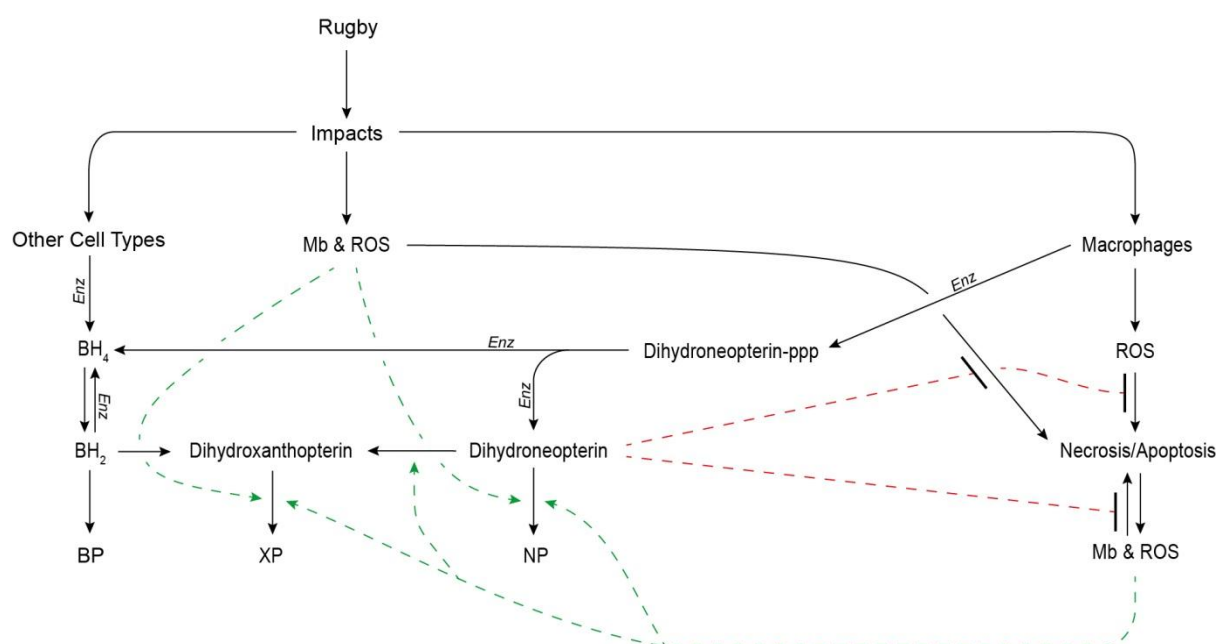
Impacts and a combination of aerobic and anaerobic metabolism have been shown to induce cells of monocyte lineage to produce dihydroneopterin and NP through oxidation in a ratio (3:1) similar to that previously reported (Fuchs *et al.*, 1989b). Oxidant production appears to be the rate limiting factor (data not shown), while the increase in dihydroneopterin is indicative of an acute inflammatory reaction. Additionally, the significant difference in the post-game concentrations between games suggests some induce a greater inflammatory reaction that could be attributed to unmeasured factors such as impact force and time spent at different running intensities. However, due to post-game concentration being significantly affected by the pre-game, those players who enter each game with an over activated inflammatory system are more likely to have a greater reaction to the stress.

The post-game concentrations in markers of oxidative stress (NP, BP and XP) were different between games, an indication that rugby induces variable amounts of oxidative stress on a game by game basis. Meanwhile, an adaptation process to the level of oxidative stress appears to be evident when more games are played as indicated by the smaller increase in NP. The consistent elevation of NP but variable response of BP and XP however, suggests different ROS are responsible for the oxidation of their precursors, or dihydroneopterin oxidation to NP occurs more readily. Because oxidative stress contributes to muscle damage (Aoi *et al.*, 2004), the measurement of NP, BP and XP may provide a new means of quantifying total skeletal muscle stress in a non-invasive manner. Traditionally analysed by serum markers like malondialdehyde, catalase, reduced and oxidized glutathione, and

superoxide dismutase (SOD) (Cardoso *et al.*, 2012), this study's simple high performance liquid chromatography (HPLC) and enzyme linked immunosorbent assay (ELISA) urine analysis can simultaneously measure muscle damage and oxidative stress to give an overall assessment.

The significant measureable increase in total BP is indicative of rugby inducing the biosynthesis of the potent endogenous vasodilator NO and monoamine neurotransmitters which is in accordance with other exercise protocols (Green *et al.*, 2004). A cofactor in several reactions, BH<sub>4</sub> is produced by several cell types and plays a significant role in vasodilation, reward-motivated behaviour and the fight or flight response (Daubner *et al.*, 2011; Kwon *et al.*, 1990). The significant relationship between pre and post-game suggests those entering a game with a high concentration will finish with a higher concentration, while it seems the impacts and intermittent nature of the game causes activation of the flight or fight response that enables player's bodies to react and perform as required. Moreover, the significant change in BP following games three and four only may be a reflection of the activity of BH<sub>2</sub> reductase and the salvage pathway (Crabtree *et al.*, 2009), or the variable level of impact induced oxidative stress across the games.

The correlation that exists between Mb and total NP indicates activation of the acute phase inflammatory reaction in response to impact induced integrity loss of the muscle cell membrane. A cascade of events may ensue that can contribute to the abundance of ROS and the further oxidation of muscle cell membrane lipids and activation of enzymes responsible for apoptosis and necrosis (Fig. 7.7) (Anzai *et al.*, 2000; Duncan and Jackson 1987; Goll *et al.*, 2003). The resulting cell death could subsequently release Mb from neighbouring undamaged muscle cells resulting in a positive feedback loop. This theory could provide more information about the effect of impact sports such as rugby, rugby league, American football and boxing, on the level of stress an athlete may experience from impact related muscle cell lysis. However, dihydroneopterin, a potent antioxidant (Giese *et al.*, 2001a), in combination with other known antioxidants, could minimize the effect of lipid peroxidation by ROS following muscle damage and cell death and consequently cause inhibition of the feedback loop (Fig. 7.7).



**Figure 7.7.** A diagram showing the major cascade of reactions discussed in this article following rugby related impacts. Green dashed lines represent a positive effect on the reaction while red lines represent an inhibition of the selected reaction. Dihydroneopterin-ppp, dihydroneopterin tri-phosphate; Enz, enzymatic reaction.

## Perspective

The rate of dihydroneopterin oxidation by Mb and iron identifies its capacity to induce oxidative stress following muscle damage in rugby. The correlation between impacts and myoglobin corroborates previous work that identified them as the source of elevated muscle damage (Smart *et al.*, 2008), while it seems the increase in selected urinary pterins by rugby related impacts provides more relative stress information about muscle damage, oxidative stress, inflammation, vasodilation and monoamine neurotransmitter synthesis in the sport. This type of analysis provides a new understanding of the stresses involved in professional rugby union that is more often analysed for markers of inflammation and immune system function. Considering oxidative stress plays a significant role in muscle stress, in conjunction with muscle damage, inflammation and indirect measurement of adrenaline secretion, a greater understanding and appreciation of the stresses involved in one of the most physically

demanding sports is presented. These values can provide future reference for other studies on rugby union and other sports to compare levels of stress against and gauge the intensity of the exercise/activity.

# Chapter 8

## Global Positioning System Technology Does Not Correlate With Cardiovascular Stress Measured as Urinary NT-proBNP in Professional Rugby Union

### Abstract

Rugby union is a physical, high force collision sport where cardiovascular stress assessment is often neglected but which is an important aspect of physiological stress management of a player. The relationship between global positioning system (GPS) and cardiovascular stress quantification through biochemical analysis is not known. Urine samples (14 in game one and 13 in game two) were collected from professional rugby players before, immediately after and 36 hours post in two consecutive games and analysed for NT-proBNP by ELISA. Comparison with microtechnology and video analysis was conducted. There was a significant increase in urinary NT-proBNP for game one ( $31.6 \pm 5.4$  to  $53.5 \pm 10.8$  pg/mL) and game two ( $35.4 \pm 3.9$  to  $49.8 \pm 11.7$  pg/mL) that did not correlate with the number of impacts, total distance covered, distance covered at pre-determined velocity bands or player-load. There was also a large inter-individual variation in NT-proBNP among players ( $p < 0.001$ ). The increase in NT-proBNP indicates professional rugby union induces an increase in cardiovascular stress. There was a very weak correlation with GPS/impacts and urinary NT-proBNP which suggests GPS technology is an inaccurate quantification of cardiovascular stress in professional rugby. Urinary NT-proBNP may provide a more simple, non-invasive and accurate measurement of cardiovascular stress that can be used in conjunction with GPS to quantify workload in professional athletes.

### 8.1 Introduction

Cardiovascular stress assessment is often neglected when monitoring the internal load of athletes despite being a pertinent marker of exercise performance, cardiovascular fatigue and subsequent performance potential. As a sport of intermittent nature (Cunniffe *et al.*, 2009) dominated by high force impacts (Hendricks *et al.*, 2012), rugby union is a team sport that may induce substantial changes in cardiovascular stress.

Global positioning system (GPS) is typically used in conjunction with game statistics (distance covered, number of impacts, player-load and ball possession) to gauge the amount of aerobic and anaerobic work completed by an athlete. Time spent and distance covered in pre-determined velocity bands classified by professional teams and manufacturing companies are the primary measure to assess the contemporary demands of professional sport (Coughlan *et al.*, 2011). This method of analysis does not take into consideration the individual variation in player threshold and capacity which makes it inaccurate when attempting inter-athlete comparisons using “generic” or global velocity bands. Biomarkers however, have been used extensively in sporting (Lindsay *et al.*, 2015c) and clinical contexts (Venet *et al.*, 2011) to accurately provide quantitative individualized assessment used to identify and treat athletes and patients accordingly. Certain issues arise through administering these measures practically, and there is equivocal evidence that surrounds their use as a means to monitor the internal training response within intermittent sports as a measure of non-functional overreaching (NFO) (Halson 2014). With changes in biomarker concentration considered the gold standard in intensive care units for diagnosis and recovery (Han *et al.*, 2009), there is a consensus for their consideration and utilization in high intensity sport where they might provide a more precise overview of athlete workload in combination with traditional GPS software.

N-terminal prohormone of brain natriuretic peptide (NT-proBNP), the cleaved inactive fragment of brain natriuretic peptide (BNP) synthesized by cardiac myocytes (Hall 2004) and fibroblasts (Tsuruda *et al.*, 2002) in response to ventricular wall tension/stress, has been shown to rise in patients with cardiac dysfunction (Lubien *et al.*, 2002) and healthy individuals following strenuous endurance exercise (Corsetti *et al.*, 2012). It has been suggested as a reliable and sensitive marker of cardiovascular stress following exercise that has potential cyto-protective and growth regulating effects (Banfi *et al.*, 2010; Scharhag *et al.*, 2005). The origin of its release remains equivocal following exercise with some instances suggesting myocardial wall necrosis leading to its secretion (Ohba *et al.*, 2001) which is reinforced by the simultaneous detection of cardiac troponin T (cTnT) (Ohba *et al.*, 2001; Rifai *et al.*, 1999). In contrast, other studies have not detected this measureable change (Scharhag *et al.*, 2005) suggesting the release may be due to an increase in cardiac myocyte membrane permeability (Scharhag *et al.*, 2005).



Additionally, the effect of impact trauma may induce further myocardial necrosis or ultra-structural cardiac muscle damage confounding the effect of wall stress on NT-proBNP secretion. With approximately 800 and 1275 combined impacts recorded for backs and forwards respectively in a professional rugby game (Cunniffe *et al.*, 2009), there is a possibility of impact related cardiac wall stress and lesion or necrosis occurring. With blunt trauma to the chest shown to cause cardiac damage in several sports including rugby (Moore 2001), high values without the signs of heart failure may be a sign of cardiac fatigue and associated with decreased exercise performance (Mattsson *et al.*, 2010). This may establish NT-proBNP as a potential prognostic marker of cardiovascular stress assessment that could be used to provide player workload quantification in conjunction with GPS statistics.

The aim of this study is to measure the level of cardiovascular stress following two consecutive games of professional rugby union and determine whether GPS statistics (distance covered, number of impacts and player-load) can explain the hypothesized rise in urinary NT-proBNP.

## 8.2 Methods

### *Experimental Approach to the Problem*

Urine samples were collected pre-game (approx. 120mins), immediately post-game ( $38.1 \pm 17.3$  mins and  $30.4 \pm 19.4$  mins for games one and two respectively) and 36 hours post-game for two consecutive games.

### *Urine and saliva collection*

Refer to Materials and Methods (Chapter 2.2).

### *Subjects*

Thirty-seven professional rugby players volunteered for the study. Of these volunteers, 23 were selected for each game. Of those selected, 14 provided pre, post and 36 hours post-game samples for game one, and 13 players for game two. Table 8.1 shows details of the subjects enrolled in the study. The experimental protocol was approved by the University of Canterbury Human Ethics Committee, Christchurch, New Zealand and all subjects were

informed (Appendix M) of the risks involved in the study before their written consent was obtained (Appendix L).

*GPS, Video-Analysis and Sample Analysis*

Refer to Materials and Methods (Chapter 2.2)

**Table 8.1.** Subject characteristics. Data is mean  $\pm$  SD.

Age (years)	26.0 $\pm$ 3.5
Height (m)	1.86 $\pm$ 0.07
Weight (kg)	104.5 $\pm$ 9.3

*Statistical Analysis*

The effect of game variables (number of impacts and distances covered) on the change in concentrations of NT-proBNP was tested in a linear mixed effects model fitted with restricted maximum likelihood, conducted in the lme4 package (Bates *et al.*, 2014) in R version 3.1.1 (Team 2014). P values for coefficients of fixed effects were calculated using Satterthwaite's method of denominator synthesis, conducted in the lmerTest package (Kuznetsova *et al.*, 2013) for R. NT-proBNP was analysed as the response variable. The fixed predictors in each model were time (pre vs. post-game vs. 36 hours post-game), the number of impacts in the game sustained by the player (which was replicated at the level of games per player), and the total distance and distance at pre-specified speed bands. Player identity was included as crossed random effects to account for the non-independence of marker measures from each player, and the potential for some games to have different effects on all players to others. Although there is no true replication of players (multiple measures are pseudo replicates) and individual levels of these factors cannot be compared, we tested whether there was significant overall variation explained by the random effects using a likelihood ratio test. We began with a full model that included all fixed and random effects, and all possible interactions among fixed effects. The shared variance was not attributed to any predictor therefore to understand the extent to which variance may have been shared we tested each effect independently. The

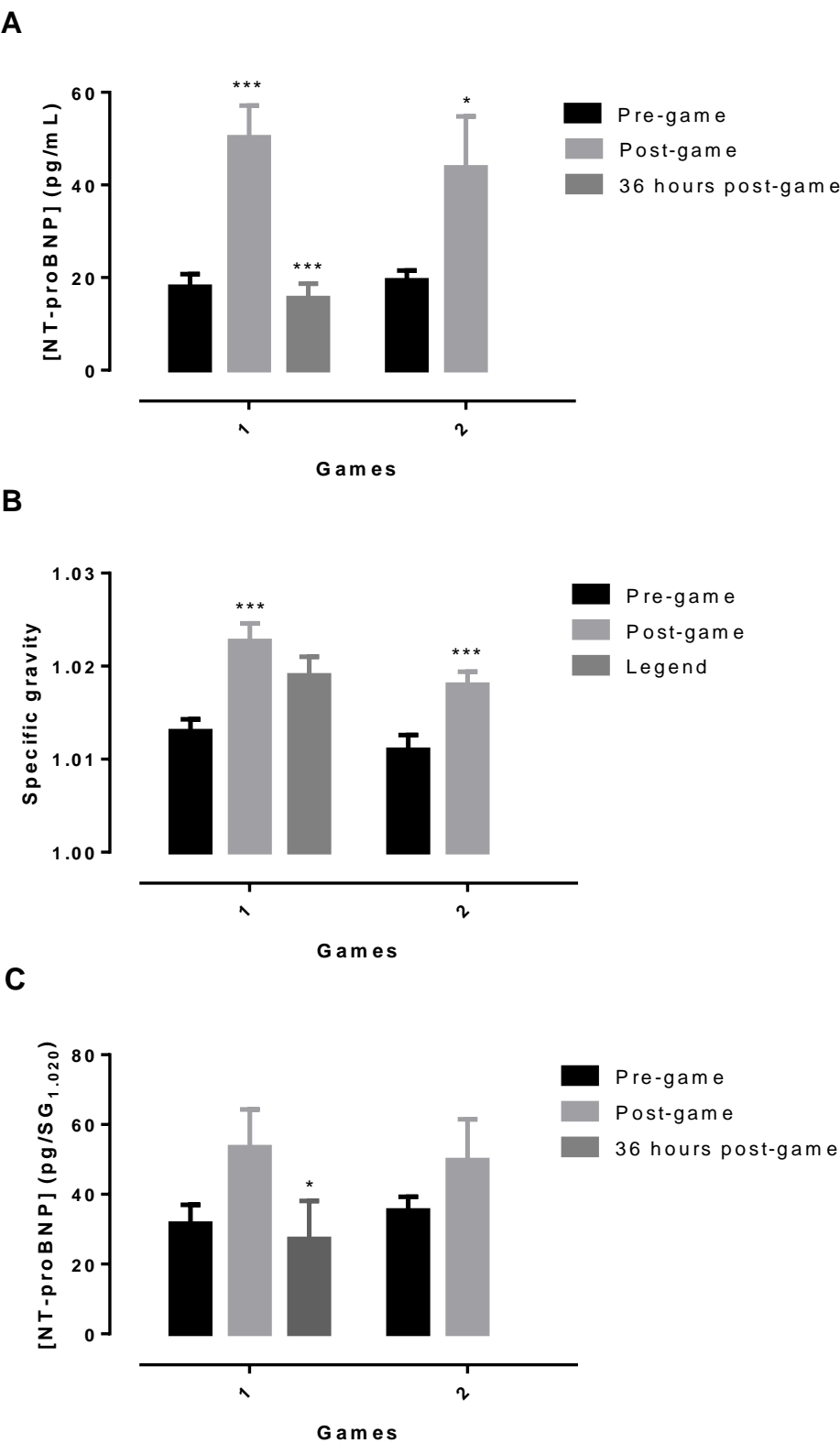
relationship between post-game percentage changes in NT-proBNP, impacts and GPS data was analysed by Spearman's rank correlation and represents samples from both games. Percentage changes were chosen to represent the actual physiological effect of the games irrespective of the pre-game concentration which may be affected by multiple variables. All samples were analysed in duplicate and significance was measured as \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ .

### 8.3 Results

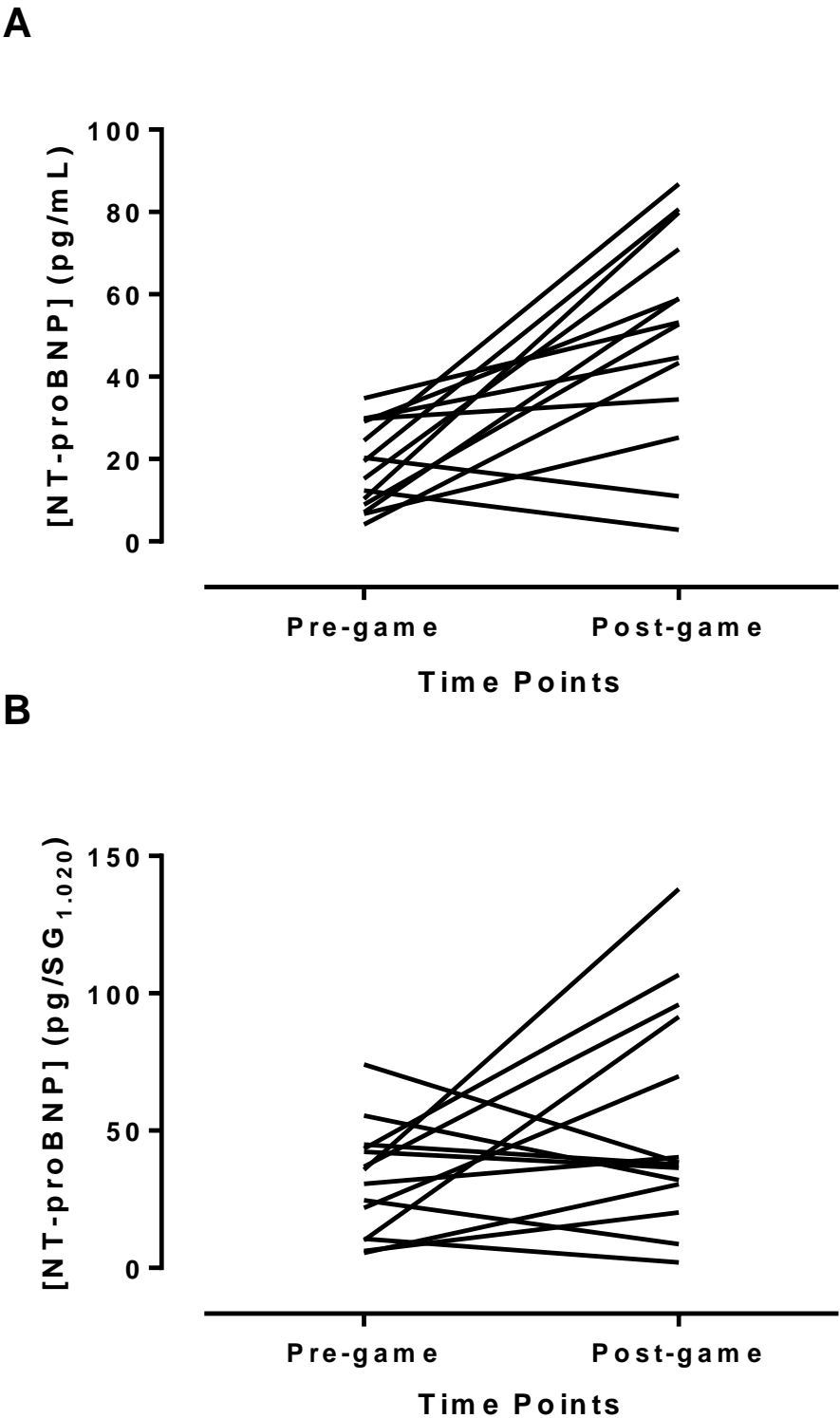
Urinary NT-proBNP concentrations when not corrected for urine volume increased significantly ( $p < 0.001$ ) from pre to post-game and decreased significantly ( $p < 0.001$ ) from post-game to 36 hours post-game (Fig. 8.1A). There was no difference ( $p > 0.05$ ) between the response of NT-proBNP between the games or players (Fig. 8.2A and 8.3A), nor did impacts, player-load or GPS data have any effect on the percentage change in urinary NT-proBNP ( $p > 0.05$ ).

Urinary specific gravity (SG) increased significantly ( $p < 0.001$ ) in game one and two from pre to post-game (Fig. 8.1B). When urine volume was corrected for, there was a suggestion professional rugby causes an increase ( $p = 0.066$ ) in urinary NT-proBNP which may be in response to the distance covered above 20 km/h ( $p = 0.0856$ ). There was also a significant decrease ( $p = 0.017$ ) from post to 36 hours post in game one (Fig. 8.1C), as well as a significant difference ( $p < 0.05$ ) in the response of players (Fig. 8.2B and 8.3B).

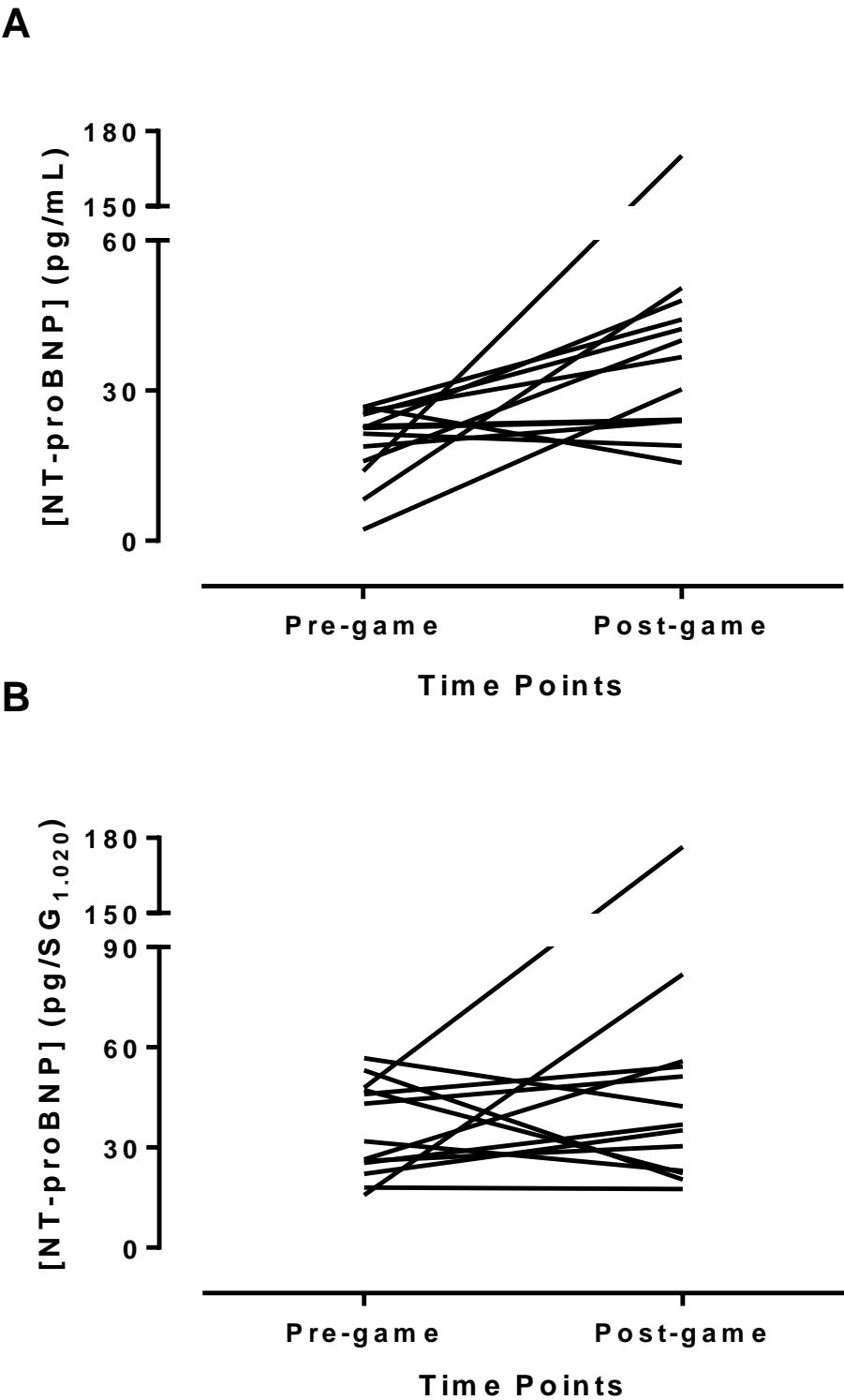
Analysis showed there was no difference between game time, number of impacts, player-load, total distance covered, or distance covered above specific velocity bands between games one and two (Table 8.2.). There was also no correlation between the pre to post percentage change in urinary NT-proBNP when corrected for urine volume and any game statistic (Table 8.3.). Individual velocity band analysis however, identifies the strongest correlation with distance covered above 7 km/h which becomes smaller with higher velocity.



**Figure 8.1.** Urinary NT-proBNP concentrations when not corrected for volume (A), urinary SG (B), and NT-proBNP corrected with SG (C). Data is presented as mean  $\pm$  SEM.



**Figure 8.2.** Individual urinary NT-proBNP response for game one when not corrected for volume (A) and when corrected with SG (B).



**Figure 8.3.** Individual urinary NT-proBNP response for game two when not corrected for volume (A) and when corrected with SG (B).

**Table 8.2.** Average GPS data and game statistics (mean ± SD).

Game	Game Time	Total Distance covered (m)	Distance (>7km/h)	Distance (>16km/h)	Distance (>20km/h)	Distance (>25km/h)	No. of impacts	Player Load
1	58.9 ± 33.7	4212 ± 2242	2685 ± 1481	827 ± 678	270 ± 174	46 ± 45	21.3 ± 13.4	460 ± 218
2	59.1 ± 27.5	4437 ± 2226	2489 ± 1329	578 ± 376	189 ± 169	26 ± 36	26.8 ± 13.5	479 ± 218

**Table 8.3.** Correlations between the pre to post-game percentage change of NT-proBNP/SG<sub>1.020</sub> with GPS data and game statistics.

		No. of impacts	Player Load	Total Distance covered (m)	Distance (>7km/h)	Distance (>16km/h)	Distance (>20km/h)	Distance (>25km/h)
NT-proBNP % change	p	0.263	0.301	0.427	0.131	0.183	0.317	0.790
	cc	0.223	0.207	0.159	0.298	0.264	0.200	0.054

### 8.4 Discussion

The aim of this study was to quantify the level of cardiovascular stress following professional rugby and whether GPS statistics could explain the hypothesized increase in NT-proBNP. The results indicate professional rugby union causes a significant increase in cardiovascular stress which is in accordance with previous exercise studies (Banfi *et al.*, 2010; Mattsson *et al.*, 2010). The return to pre-game levels 36 hours post-game signifies complete cardiovascular recovery and the unlikelihood of permanent cardiac damage. This is similar to the echocardiographic imaging and magnetic resonance imaging of marathoners where myocardial overstimulation rather than cardiac damage arose (Hanssen *et al.*, 2011). Regardless of whether this increase is a result of myocardial wall necrosis or reversible injury of the cardiac myocytes from wall stress/increased membrane permeability (Rifai *et al.*, 1999), the increase in urinary NT-proBNP suggests the intermittent nature and impacts associated with professional rugby union induces cardiovascular stress.

The non-significant relationship between all game statistics with changes in NT-proBNP suggests that GPS statistics and video analysis may not be an accurate measure of cardiovascular stress in rugby union. Or, NT-proBNP is not a good indicator of information gathered from GPS and video analysis. The non-significance between NT-proBNP and distance covered suggests the aerobic and anaerobic activity completed in these two games of rugby union may not have been sufficient to induce changes in left ventricular wall stress and stimulation of NT-proBNP secretion. Meanwhile, the non-significant effect of impacts and player-load on NT-proBNP secretion indicates the blunt trauma of repeated high force impacts involved in this study does not stimulate further cardiac wall stress or damage as previously noted (Vasudevan *et al.*, 2003). This may simply be due to the angle of impact, increased muscle mass of professional players, or the adaptation to recurrent stress. Considering an elbow blow to the anterior chest of a player during a basketball game is capable of inducing myocardial infarction (Moore 2001), all three scenarios seem like possibilities.

All taken into consideration, these observations would suggest NT-proBNP secretion as a measure of cardiovascular stress is dependent on the individual physiology of a player rather than quantitative game statistics. This is in accordance with its use as an indicator of cardiovascular fitness where trained athletes have significantly lower resting values than



sedentary controls (Banfi *et al.*, 2010). In this cohort of subjects, some are comparable to that observed in other rugby players (Banfi *et al.*, 2010) while some are analogous to sedentary controls (Cortés *et al.*, 2006). Although GPS statistics provide valuable information when monitoring and quantifying the work and corresponding workload of an athlete's external characteristics, its reliability as a measure of complete cardiovascular stress should be considered.

NT-proBNP is both an effective and accurate marker for the evaluation of heart pathologies categorized by myocardial wall stress (Palazzuoli *et al.*, 2010). The ability to quantify cardiovascular stress within four hours of the games cessation makes it an ideal addition for coaches and medical staff to prescribe recovery interventions in conjunction with the external load quantification capabilities of GPS. For assessment of cardiovascular stress and quantitative measurement of total aerobic and anaerobic work completed by a player during a game that is ultimately used to manage a players recovery and longevity, urinary NT-proBNP may be a more accurate and reliable indicator than current methods.

The immediate increase in NT-proBNP is in accordance with its elimination kinetics (Vidotto *et al.*, 2005). The 1.69 and 1.4 fold increase following game one and two respectively, is relatively small compared to previous studies that have observed an approximate two-fold increase following a marathon (Neilan *et al.*, 2006). Cortes *et al.* (2006) have shown strong correlations between plasma and urinary NT-proBNP in heart failure patients suggesting the changes observed in mountain marathons however (Banfi *et al.*, 2010) is quite comparable. Meanwhile, ultra-endurance exercise has shown median values increasing by 200 ng/L (Scharhag *et al.*, 2005) suggesting professional rugby union is not comparable in cardiovascular intensity, nor is it above the pathological threshold of 125 pg/mL (Banfi *et al.*, 2010). Participants in this study were all below this threshold barring two individuals who reached values of 136 and 176 pg/mL in different games. This may signify considerable cardiovascular damage or stress that could lead to cardiac fatigue, NFO and in severe cases result in later life arrhythmias or development of scattered fibrosis and cardiac scarring (Breuckmann *et al.*, 2009). More importantly, Cortes *et al.* (2006) proposed a cut-off value of 94 pg/mL for heart failure patients which was present in four subjects across the games. While this is most likely a cyto-protective effect and control mechanism for relieving wall tension (Scharhag *et al.*, 2005), the values represent considerable cardiovascular stress.

Further possible measurements for cardiovascular stress following exercise may include urinary pH, urinary lactate measurement and heart rate variability. While all provide relevant information, urinary pH can be affected by several other charged compounds other than lactate, while urinary lactate has restrictions that include a delay in the peak concentration post exercise of 40 – 50 mins (Johnson and Edwards 1937). Heart rate variability meanwhile has emerged as a powerful tool for measurement of exercise stress (Morales *et al.*, 2014) although it is becoming increasingly unfavourable within elite sport given the natural day-to-day fluctuations and psychometric constraints. No research however, has examined the direct relationship with known markers of cardiovascular stress or GPS data. Therefore, urinary NT-proBNP may be a more accurate and non-invasive means of cardiovascular stress assessment in athletes.

### *Conclusion*

Professional rugby union caused significant intra- and inter-individual increases in urinary NT-proBNP corresponding to an increase in cardiovascular stress. This change did not correlate with any measure of GPS technology, nor was there a correlation with any form of impact related data.

### *Practical Applications*

The measurement of urinary NT-proBNP in a non-invasive and stress free manner may provide coaches and managers with an alternative quantification tool for the assessment of cardiovascular workload in recreational and professional athletes. This type of measurement is simple and effective at identifying intra- and inter-individual variations amongst players, and capable of providing information pertaining to workload. Whilst GPS does provide some valuable information, NT-proBNP may offer a key source of information for athlete management that is not currently considered and potentially superior for quantifying cardiovascular workload. It could also be used by coaches and medical staff to identify certain athletes at risk of cardiac damage and death, as well as a tool for assessing fitness and recovery from a game or competition

Future research requires further corroboration of the conclusions drawn from this research in order to validate the use of NT-proBNP biochemical analysis as a more accurate means of cardiovascular stress assessment and work-load. Whilst rugby union's intermittent nature

and impact nature has no correlation with cardiovascular stress, other field and contact related sports may have varying degrees of exercise-induced cardiovascular stress that may be accurately measured through microtechnology.

# Chapter 9

## Immunity, Inflammatory and Psychophysiological Stress Response During a Competition of Professional Rugby Union

### Abstract

Three specific biochemical markers were measured to provide information pertaining to the longitudinal stress response during a competition of professional rugby union. Urine and saliva were collected from 37 professional players at regular intervals throughout a 20 week professional competition. Total neopterin, cortisol and secretory immunoglobulin A were analysed using ELISA and HPLC. All markers did not change significantly when analysed as a group during the course of the season compared to baseline ( $p > 0.05$ ), although long-distance travel had a minor effects on cortisol and secretory immunoglobulin A concentrations ( $p < 0.05$ ). More importantly, a large inter-individual variation for all markers was observed ( $p < 0.001$ ). These results indicate this competition of professional rugby does not cause significant changes in psychophysiological stress. Some players however, may become more susceptible to fatigue and infection during the course of a season as a result of suppressed immunity and sustained activation of the inflammatory response.

### 9.1 Introduction

Rugby union is a physical sport (Quarrie and Hopkins 2007) that incorporates three tiers of play in the professional game in New Zealand - provincial, franchise, and international. With 40 weeks/year of competitive play incorporating weights, training sessions, and games involving repeated high force impacts (Austin *et al.*, 2011), players may be susceptible to periods of under-performance and recovery that has been observed in other high intensity sports (Cunniffe *et al.*, 2011; Gleeson *et al.*, 1999). Moreover, player requirements in the modern era are not strictly rugby related but can incorporate media and sponsorship obligations, time away from friends and family, and considerable travel which have been shown to cause under-performance in rugby and other professional sports (Du Preez and Lambert 2007).

To the best of our knowledge, the measurement and quantification of longitudinal or cumulative physiological stress in professional rugby union during a season or competition

has not been investigated efficiently. There have been a handful of studies that have utilized biochemical analysis to measure cumulative stress in team sports (Banfi *et al.*, 2006; Cunniffe *et al.*, 2011; Elloumi *et al.*, 2003; Finaud *et al.*, 2006b), however the majority of these fail to observe specific patterns due to a lack of sampling, group approaches, use of limited bio-markers, and have not considered the effect of professional sport on immune system activation or the inflammatory response.

Little is currently known about the possibility of continual activation of inflammatory pathways involved in severe intensity, high impact sports and their effect in monitoring cumulative physiological stress. There is also a lack of evidence concerning the effect of long-distance travel and positional differences in professional rugby on this response. Neopterin (NP), a product of 7,8-dihydroneopterin oxidation that is released from  $\gamma$ -interferon activated macrophages (Müller *et al.*, 1991), has been shown to be an effective marker of immune system activation and inflammation in acute exercise (Moser *et al.*, 2008). Previous evidence has demonstrated rugby union causes both an immediate increase in urinary total NP (NP + 7,8-dihydroneopterin) (Lindsay *et al.*, 2015b; Lindsay *et al.*, 2014b), whilst showing promise as an indicator of short-term cumulative stress in rugby union (Lindsay *et al.*, 2015c) and competitive bodybuilding (Lindsay *et al.*, 2014a). In conjunction with salivary immunoglobulin A (sIgA) whose concentration and secretion rates have been shown to change in response to both short and long term high intensity/season long competition and flat over-trained athletes (Budgett 1998; Gleeson *et al.*, 1999), and cortisol which is regularly measured for gauging the psychophysiological state of an athlete (Morgan 2011) and whose concentration increases in stale, underperforming and over-trained athletes (Urhausen *et al.*, 1998b), NP may provide some valuable information pertaining to rugby specific immune system activation during a competition.

The aim of this study is to therefore monitor changes in immune system function, inflammation and psychophysiological stress in a non-invasive manner in professional rugby. It intends to provide information concerning group, positional and individual changes in physiological stress to quantitatively identify the longitudinal response to a physical impact sporting competition.

9.2 Methods

*Subjects*

Thirty-seven professional rugby players from one team who competed in the 2014 Super 15 competition (franchise) volunteered for the study. Details of subjects enrolled are shown in Table 9.1. Of the 37 subjects, 23 were selected per game, while the remaining players played club rugby. The experimental protocol was approved by the University of Canterbury Human Ethics Committee, Christchurch, New Zealand and all subjects were informed (Appendix M) of the risks involved in the study before their written consent was obtained (Appendix L).

**Table 9.1.** Subject characteristics. Data is mean ± SD.

Age (years)	26.0 ± 3.5
Height (m)	1.86 ± 0.07
Weight (kg)	104.5 ± 9.3

**Experimental Protocol and Sample Collection**

Urine and saliva samples were collected at regular intervals (Table 9.2) from each subject throughout the 20 week regular season between 8:30 – 10am on a Monday morning to avoid dietary and circadian influences. Between 25 and 35 subjects provided samples at each collection time point. Collection was subject to player injury and individual training protocols that may have prevented them from being present at the training facilities where collection took place.

*Urine and saliva collection*

Refer to Materials and Methods (Chapter 2.2)

*HPLC, Sample Preparation and Analysis and Specific Gravity*

Refer to Materials and Methods (Chapter 2.2)

**Table 9.2.** Timing of urine collection for total neopterin and saliva collection for cortisol and sIgA.

Testing (T1-T12)		Week	Description
N & C	sIgA		
T1	T1	- 4	Baseline – before any contact
T2	T2	- 2	Following pre-season internal trial – first contact
T3	T3	2	Game one of the regular season
T4	-	3	Game two of the regular season
T5	T4	4	Game three of the regular season
T6	T5	7	Game five which was preceded by a bye
T7	T6	10	Return from 2 games in South Africa – 7 games played
T8	-	12	Coming off a bye week – 8 games completed
T9	T7	13	Game 9 of the regular season
T10	T8	17	Return from a 3 week international series – 13 games played
T11	T9	18	Game 14 of the regular season
T12	T10	19	Game 15 of the regular season

\* N, neopterin; C, cortisol

### *Statistical Analysis*

The effect of cumulative change in concentrations of each marker after it was log transformed was tested in a linear mixed effects model fitted with restricted maximum likelihood, conducted in the lme4 package (Bates *et al.*, 2014) in R version 3.1.1 (Team 2014). P values for coefficients of fixed effects were calculated using Satterthwaite's method of denominator synthesis, conducted in the lmerTest package (Kuznetsova *et al.*, 2013) for R. Each marker

was analysed as the response variable in a separate model. The fixed predictor in each model was time, forwards vs. backs, those subjects who travelled to South Africa for three weeks vs. those that did not, and those subjects who played in a three week international series vs. those that did not. Each fixed factor was analysed independently of the other. Player identity was included as crossed random effects to account for the non-independence of marker measures from each player. As there is no true replication of players (multiple measures are pseudo replicates), individual levels of this factor could not be compared, so we tested whether there was significant overall variation explained by the random effects using a likelihood ratio test. Significance was set at  $p < 0.05$  and data is presented as mean  $\pm$  SEM.

### 9.3 Results

During the competition, the team played 16 games finishing with a record of 11 wins and 5 losses. They began the first half of the season with a 5 - 3 record and finished with a 6 - 2 record. Salivary cortisol for the group did not significantly change over time compared to the beginning of the competition, except for a significant increase during week 3 ( $p = 0.002$ ,  $n^2_{\text{partial}} = 0.881$ ), decrease following the return from South Africa (week 10) ( $p = 0.031$ ,  $n^2_{\text{partial}} = 0.537$ ), and an increase ( $p = 0.017$ ,  $n^2_{\text{partial}} = 0.601$ ) during week 18 (Fig. 9.1A) compared to baseline. There were also no differences between the forwards and backs (Fig. 9.1B). There was however a significant difference ( $p < 0.001$ ,  $n^2_{\text{partial}} = 1.52$ ) in concentration between the participants who travelled to South Africa compared to those that did not upon their return (Fig. 9.1C).

Urinary total NP analysis found no significant changes throughout the competition (Fig. 9.2). Group analysis showed an observable upward trend that was not statistically significant during the 20 week competition (Fig. 9.2A), while travelling to South Africa (Fig. 9.2C) did not cause any significant changes in total NP. The only significant difference measured ( $p = 0.033$ ,  $n^2_{\text{partial}} = 0.271$ ) was that between forwards and backs following the pre-season internal trial game (Fig. 9.2B).

Group sIgA concentration (Fig. 9.3A) was shown to increase during the competition with significant differences measured following the international break ( $p = 0.003$ ,  $n^2_{\text{partial}} = 0.272$ ) and during week 19 ( $p = 0.009$ ,  $n^2_{\text{partial}} = 0.535$ ) compared to baseline. There was no difference between forwards and backs (Fig. 9.3B) and a significantly higher concentration ( $p$

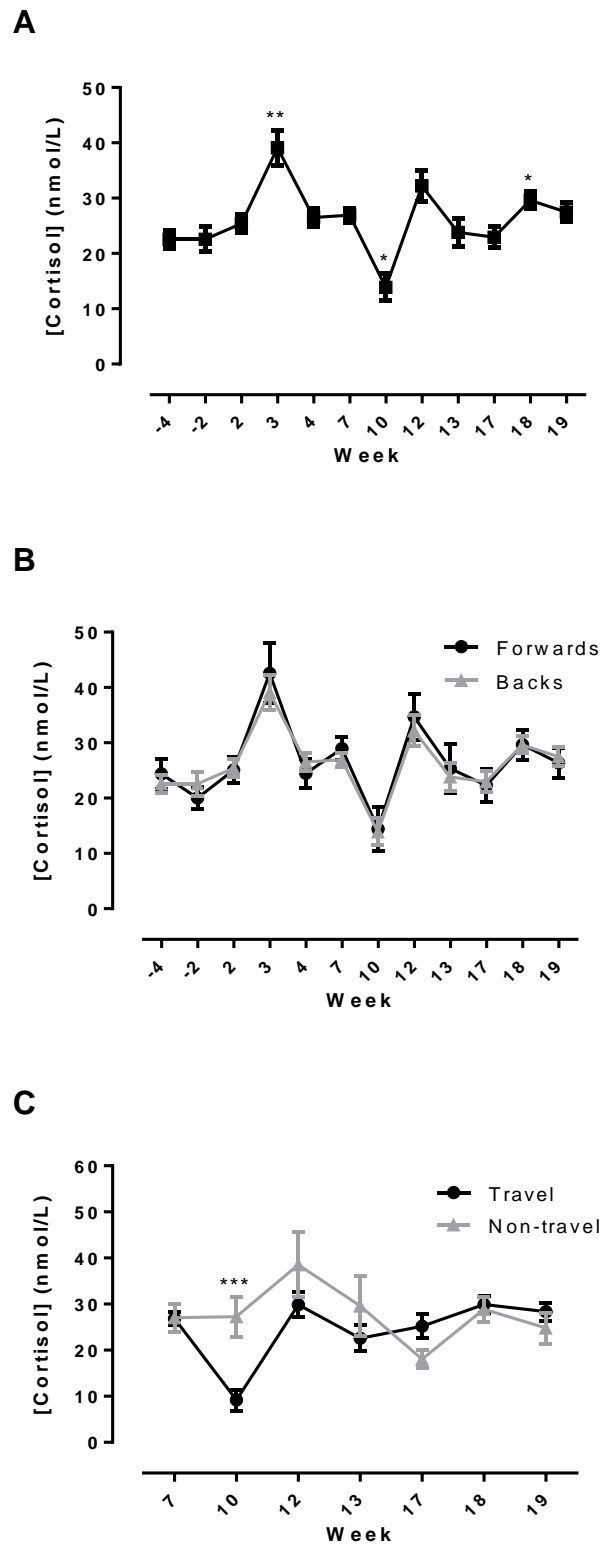


= 0.042,  $n^2_{\text{partial}} = 0.167$ ) in those participants who travelled to South Africa during week 19 (Fig. 9.3C).

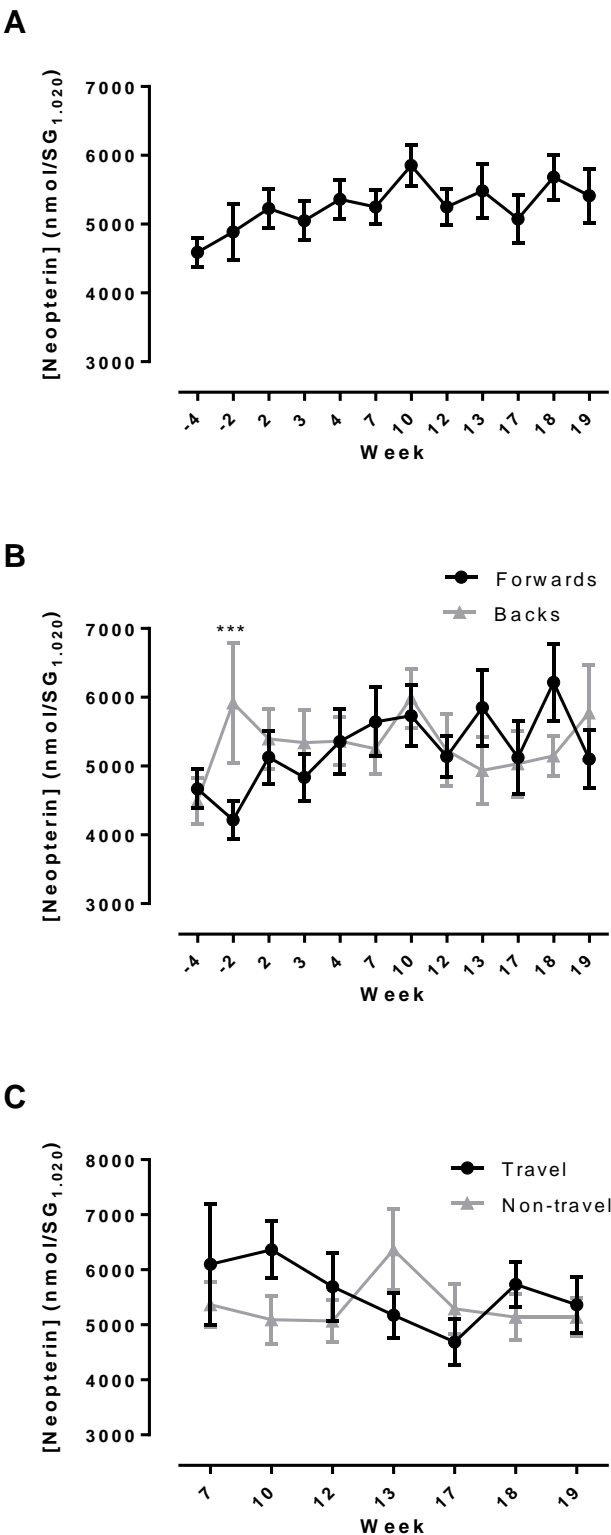
sIgA secretion rate showed a very similar pattern (Fig. 9.4A) with significantly higher secretion rates observed following the international break ( $p = 0.05$ ,  $n^2_{\text{partial}} = 0.654$ ) and during week 19 ( $p = 0.033$ ,  $n^2_{\text{partial}} = 0.469$ ) compared to baseline. There were no differences between forwards and backs (Fig. 9.4B) or between those participants who travelled to South Africa compared to those that did not (Fig. 9.4C).

When participants were analysed individually (not the same two subjects but selected subjects who showed variable differences), all markers showed significant ( $p < 0.001$ ) differences between their responses (Fig. 9.5). Whilst salivary cortisol (Fig. 9.5A) and urinary total NP (Fig. 9.5B) for one subject show a relatively unchanged response over the competition, another subject had spikes and troughs (cortisol) or continual elevation (NP) above their initial baseline. The sharp drop at time point 10 observed for total NP coincided with a three week rest during the international break. When analysing immune system status, one individual showed an increase in function over time, while another steadily decreased (Fig. 9.5C) with periods of suppression and restoration (Fig. 9.5D).

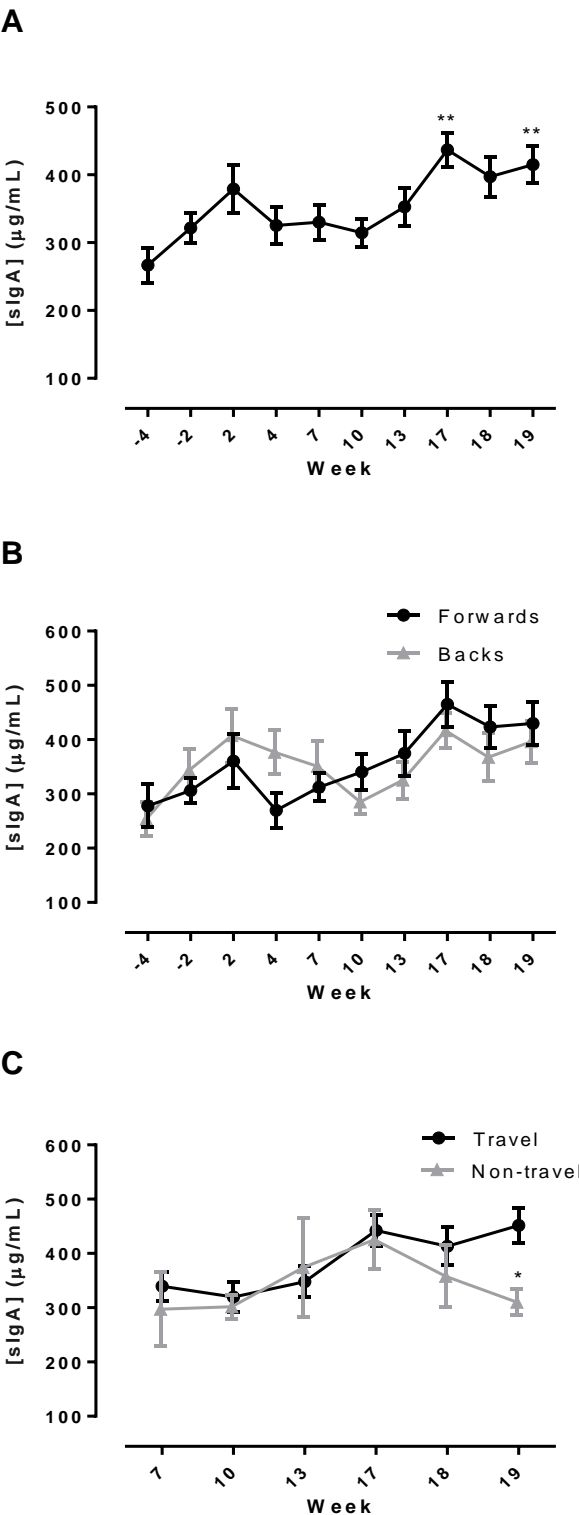
When participants were analysed individually, all markers showed significant differences between their responses ( $p < 0.001$ ). Figure 9.5 represents selected individuals within the group who showed signs of non-functional overreaching (NFO)/overtraining syndrome (OTS) compared to those who did not. Whilst salivary cortisol (Fig. 9.5A) and urinary total NP (Fig. 9.5B) for one subject show a relatively unchanged response over the season, another subject had spikes and troughs (cortisol) or continual elevation (NP) above their initial baseline. The sharp drop at time point 10 observed for total NP coincided with a three week rest during the international break. When analysing immune status, one individual showed an increase in function over time, while another steadily decreased (Fig. 9.5C) with periods of suppression and restoration (Fig. 9.5D).



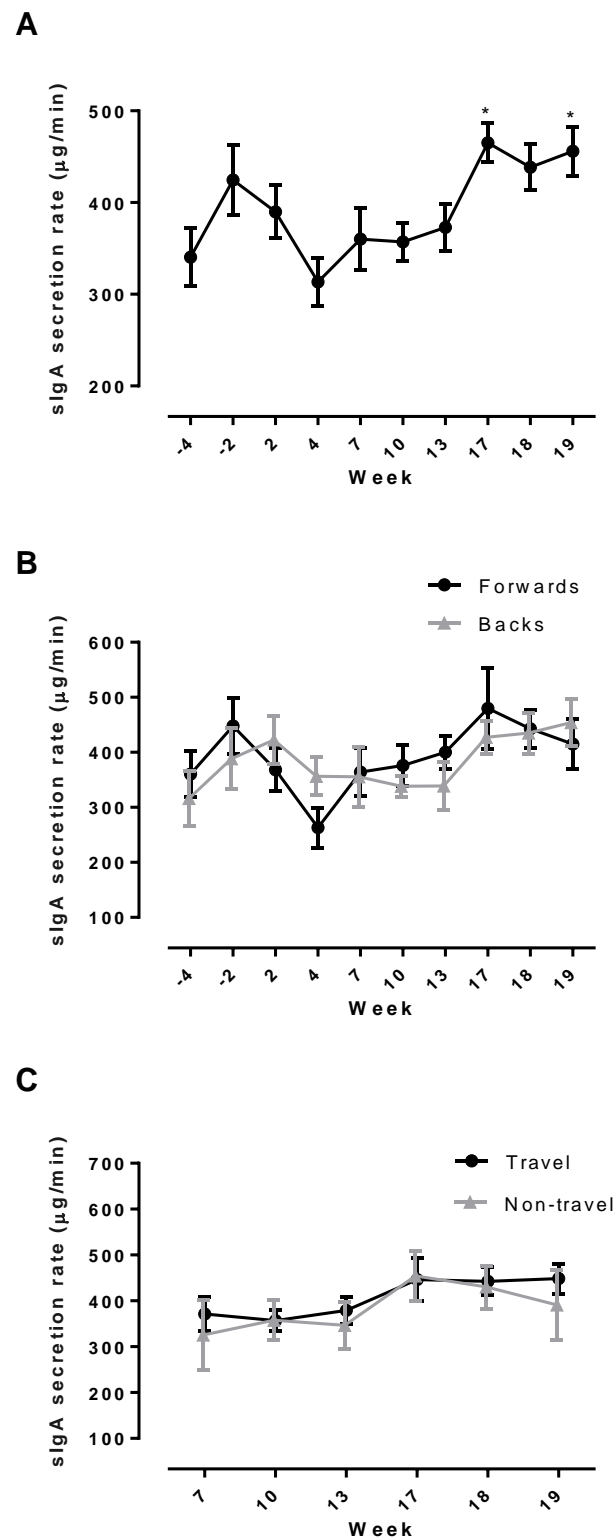
**Figure 9.1.** Salivary cortisol for the group (A), forwards vs. backs (B) and players who travelled to South Africa vs. those that did not (C). Data is presented as mean  $\pm$  SEM. (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).



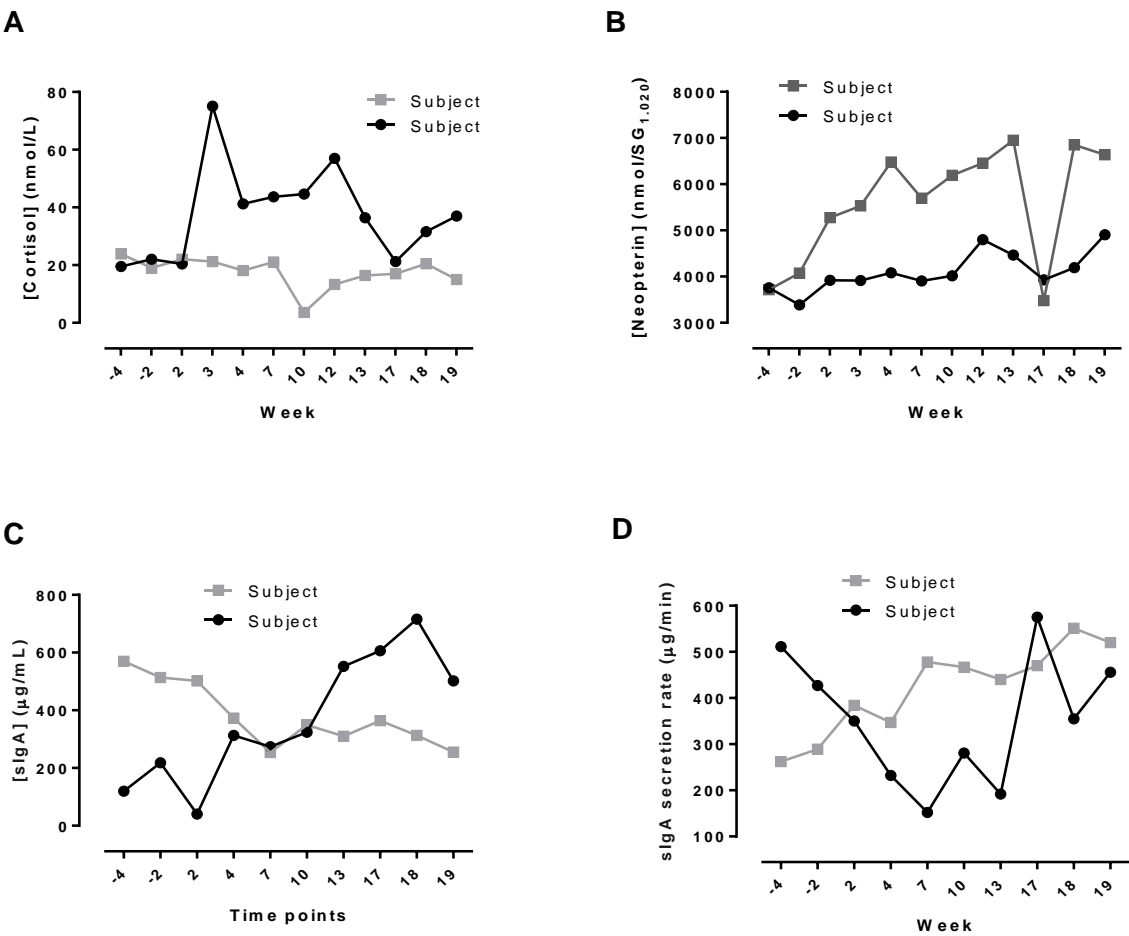
**Figure 9.2.** Urinary total neopterin for the group (A), forwards vs. backs (B) and players whotravelled to South Africa vs. those that did not (C). Data is presented as mean  $\pm$  SEM. (\*\*\*)  $p < 0.001$ ).



**Figure 9.3.** sIgA concentration for the group (A), forwards vs. backs (B) and players who travelled to South Africa vs. those that did not (C). Data is presented as mean  $\pm$  SEM. (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).



**Figure 9.4.** sIgA secretion rate for the group (A), forwards vs. backs (B) and players who travelled to South Africa vs. those that did not (C). Data is presented as mean  $\pm$  SEM. (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).



**Figure 9.5.** Longitudinal changes in cortisol (A), total neopterin (B), sIgA concentration (C) and secretion rate (D) of selected individuals who responded differently.

**9.4 Discussion**

This study provides clear and concise evidence of the longitudinal stress encountered during this professional rugby competition at both an individual and group context. Biochemical analysis using haematological (Banfi *et al.*, 2006) and immuno-endocrine (Cunniffe *et al.*, 2011) parameters of athletes in team sports has been used to identify longitudinal changes in physiological stress (Hoffman *et al.*, 2005). Problems that have arisen from this type of analysis revolve around the level of sampling and methodological approach that we feel does

not justify an adequate understanding of physiological assessment (Elloumi *et al.*, 2003; Finaud *et al.*, 2006b), although some studies have sampled up to 20 times (Cormack *et al.*, 2008). Furthermore, analysis has normally been conducted during a short training block (Coutts *et al.*, 2007) or entire year (Cunniffe *et al.*, 2011) where unfortunately it cannot be utilized to its fullest benefit. While this can identify trends, it does not take into account the individual week by week response of an athlete throughout an entire competition/season where it is most pertinent for understanding and observing the potential change. The type of analysis performed in this study in conjunction with traditional performance measures may become a useful predictive tool for identifying certain players at risk of developing functional overreaching (FO), NFO or OTS.

The stress markers putatively indicative of inflammation and immunity were not affected substantially at a population level by professional rugby which contrasts the findings of a similar study in professional rugby (Cunniffe *et al.*, 2011) and Australian Rules Football (Cormack *et al.*, 2008). It is however in accordance with other team sports such as American Football (Hoffman *et al.*, 2005; Kraemer *et al.*, 2013) with differences potentially attributed to the different physical intensities of the sports, the level of competition, or the type of analysis/sample collection protocol which is known to affect concentrations (Strazdins *et al.*, 2005). The analysis does provide a key insight into the individual player response variability and the capability of biochemical analysis to highlight this difference. While some players (a mixture of positions and ability) present the ability to recover week by week, there is a suggestion that others may be failing to recover as a result of the stresses imposed during a competition of professional rugby and the associated training and commitments.

Inflammatory markers are typically used for quantification of the acute phase response to exercise (Cunniffe *et al.*, 2010). Their effectiveness as a marker of longitudinal stress in a professional contact sport however, has not been explored in depth (Cunniffe 2012). Neopterin's observable increase as a group indicates there is increased immune activation during the competition. This could potentially correspond to any form of under-performance (Meeusen *et al.*, 2013) which contradicts the response of cortisol and sIgA, or is simply a coping mechanism used to alleviate the week by week stress of the high force impacts. This is in contrast to a decrease in C-reactive protein (CRP) over the course of a year in professional rugby (Cunniffe 2012) which may be a result of different intensities between the

groups, or the small number of time points tested in the latter study (seven). Total NP's use in the clinical and sporting field as an indicator of an acute phase inflammatory response has been well documented (Fuchs *et al.*, 1989a; Moser *et al.*, 2008), however this is the first study that utilizes its known function to monitor and observe longitudinal stress in any athlete. Urinary total NP may therefore provide a new angle of cumulative physiological stress assessment that has not been considered.

Moreover, while there was a lack of difference between player position and no further change between players who travelled compared to those that did not, individual total NP analysis provided a clear and resolute picture of individuals whose immune systems are increasingly over-activated. Smith (2000) suggested that “repetitive intense exercise coupled with inadequate rest causes micro-trauma to joints, muscles and connective tissue, resulting in a cytokine induced initiation of a ‘whole-body’ response involving chronic systemic inflammation that may be more related to recovery and survival than adaptation to stress”. Coupled with a sustained inflammatory response known to down regulate muscular strength (Roubenoff 2000) and physical performance (Cesari *et al.*, 2004) and used to assess disease progression (Fuchs *et al.*, 2009), inflammatory analysis through urinary total NP measurement may be a useful monitoring tool for informing team staff about players struggling to recover.

The lack of change in cortisol in conjunction with the observed increase in sIgA concentration and secretion rate may represent higher levels of immunity, a decreased susceptibility to upper respiratory infections and indicative of careful player management (work-load). This may indicate professional rugby as a whole (weights and fitness sessions interspersed with rest days culminating in a game) is of a moderate intensity which is known to increase sIgA (Klentrou *et al.*, 2002), or more than likely there is a significant adaptation process occurring. This is in contrast to research that showed no change in sIgA during a year of professional rugby (Cunniffe *et al.*, 2011). Differences may be attributable once again to sampling procedures which can be affected by collection technique (Strazdins *et al.*, 2005) or the differences in game play between northern and southern hemisphere rugby. It also suggests the pre-season intensity training responsible for preparation of professional rugby union is of a great intensity.



Travel also seems to influence the biochemical balance. Few studies have investigated the effects of long-haul travel (Bullock *et al.*, 2009), however even though professional rugby players are expected to return to training immediately, diurnal rhythm complications or “jet-lag” does not seem to have any long-term effect on the psychophysiological state of those players or the team results.

### *Conclusion*

A professional rugby competition does not cause significant group changes in selected markers of psychophysiological stress. The high inter-individual variation however shows some players are susceptible to fatigue and illness during the course of the competition, while long distance travel has significant physiological effects. Cortisol, sIgA and total NP each provide a unique insight into the longitudinal physiological stress response over the course of a competition and could therefore be used in conjunction with traditional performance measures by training, medical and coaching staff to better inform player management.

### *Practical Applications*

- Individual physiological stress assessment in professional rugby players is imperative.
- Neopterin and markers of an inflammatory response may provide useful information pertaining to longitudinal and cumulative stress associated with impact related sport.
- Longitudinal stress seems to be comparable between positions in rugby union.
- Long distance travel may not have a long-term effect on physiological stress in impact related team sports.

# Chapter 10

## Positional Demands of Professional Rugby

### Abstract

Rugby union is a physically intense intermittent sport coupled with high force collisions. Each position within a team has specific requirements which are typically based on speed, size and skill. The aim of this study was to investigate the contemporary demands of each position and whether they can explain changes in psychophysiological stress. Urine and saliva samples were collected before and after five selected Super 15 rugby games from 37 players. Total neopterin, cortisol and immunoglobulin A were analysed by SCX-HPLC and ELISA. GPS software provided distance data, while live video analysis provided impact data. All contemporary demands were analysed as events per minute of game time. Forwards were involved in more total impacts, tackles and rucks compared to backs ( $p < 0.001$ ), while backs were involved in more ball carries and covered more total distance and distance at high speed per minute of game time ( $p < 0.01$ ). Loose forwards, inside and outside backs covered significantly more distance at high speed ( $p < 0.01$ ), while there was a negligible difference with number of impacts between the forward positions. There was also minimal difference between positions in the percentage change in neopterin, cortisol and sIgA. The results indicate distance covered and number of impacts per minute of game time is position dependent whereas changes in psychophysiological stress are independent. This information can be used to adapt training and recovery interventions to better prepare each position based on the physical requirements of the game.

### 10.1 Introduction

Rugby union is a sport that is intermittent in nature and dominated by high force and frequency impacts (Austin *et al.*, 2011). Evaluation of workload has been assessed through global positioning system (GPS) technology (Cunniffe *et al.*, 2009), video analysis (Deutsch *et al.*, 2007), computerized tracking systems (Lacome *et al.*, 2014) and markers of physiological and psychological stress (Cunniffe *et al.*, 2010) to provide performance assessment, manage player recovery, and understand the demands of professional rugby. The accuracy of these quantitative measurements allows the precise monitoring of game

statistics that can be used to better adapt recovery interventions and specific training requirements.

The demands of rugby union are well documented, especially since the onset of the professional era where more time is spent in the physical preparation and skill development of players. Speed and size of players (Fuller *et al.*, 2012), work to rest ratios (Austin *et al.*, 2011; Duthie *et al.*, 2005), tackle forces (Usman *et al.*, 2011), and number of tackles and rucks (Quarrie and Hopkins 2007) have all significantly changed since the shift towards professionalism. Whether these changes in match play are related to changes in psychophysiological stress is currently unknown. Psychophysiological stress too can be used as a precise tool for managing player recovery. This type of analysis has been utilized extensively in rugby and other team sports to provide a quantitative measurement of exercise stress (McLellan *et al.*, 2010; Smart *et al.*, 2008), however it has been previously noted that the number of impacts a player experiences or distance they cover does not necessarily correlate with the total amount of stress (Lindsay *et al.*, 2014b). A combination of GPS, video and computerized analysis and psychophysiological assessment can provide an understanding of player stress to each game to better understand the contemporary demands of the game and how best to prepare an individual.

Like other team sports, specific physical characteristics are required for each position based on size, speed and skill. Studies using GPS in hockey (Gabbett 2010), rugby league (Waldron *et al.*, 2011) and Australian Rules Football (AFL) (Brewer *et al.*, 2010) have all identified position specific requirements. Similarly, rugby forwards are typically larger in size, engaged in more impacts, and cover significantly less distance, while backs are considerably lighter, involved in less impacts and cover significantly more total distance and distance at high speed (Cunniffe *et al.*, 2009; Deutsch *et al.*, 2007). A thorough understanding of the unique demands of each position is invaluable for individualized preparation.

There are several studies evaluating the demands of positions within a rugby team (Venter *et al.*, 2011). The problem with a lot of the current literature surrounding rugby union studies is the presentation of data with minimal regard to game time, while the effect these demands have on the physiological response is relatively unknown. We know the demands are dependent on the requirements of a particular position, and players are selected based on their

abilities to complete the necessities of that position. With this in mind, this study hopes to compare the positional demands of professional rugby incorporating impacts, distance and percentage changes in markers of psychophysiological stress.

## 10.2 Methods

### *Subjects*

Thirty-seven professional rugby players from a Super 15 franchise volunteered for the study. Of the 37 who volunteered, 23 were selected to play each game with 20 providing samples in game one (eight forwards, 12 backs), 16 in game two (nine forwards, seven backs), 14 in game three (seven forwards, seven backs), 17 in game four (ten forwards, 7 backs) and 14 in game five (eight forwards, six backs).

- Front Row: props and hooker ( $n = 28$ )
- Locks: locks ( $n = 11$ )
- Loose forwards: blindside and openside flanker and No.8 ( $n = 17$ )
- Inside backs: half-back, fly-half, 2<sup>nd</sup> 5/8<sup>th</sup>, centre ( $n = 21$ )
- Outside backs: wings and full-back ( $n = 27$ )

Details of subjects enrolled in the study are shown in Table 10.1. The experimental protocol was approved by the University of Canterbury Human Ethics Committee (Christchurch, New Zealand) and all subjects were informed (Appendix M) of the risks involved in the study before their written consent was obtained (Appendix L).

### *Experimental Protocol and Sample Collection*

Urine and saliva samples were collected pre-game (within 120mins) and post-game (within 60mins) for five home games.

### *Saliva and urine collection*

Refer to Materials and Methods (Chapter 2.2)

*HPLC, Sample Preparation and Analysis, Specific Gravity and Game Statistics*

Refer to Materials and Methods (Chapter 2.2)

**Table 10.1.** Subject characteristics. Data is mean  $\pm$  SD.

	Front row	Locks	Loose Forwards	Inside Backs	Outside Backs
Age (years)	26.6 $\pm$ 3.73	23.7 $\pm$ 2.14	27.0 $\pm$ 4.41	27.5 $\pm$ 2.63	25.8 $\pm$ 1.26
Height (m)	1.86 $\pm$ 0.04	2.01 $\pm$ 0.05	1.88 $\pm$ 0.04	1.81 $\pm$ 0.02	1.89 $\pm$ 0.05
Weight (kg)	112.1 $\pm$ 5.14	112.3 $\pm$ 3.51	106.5 $\pm$ 2.30	92.9 $\pm$ 3.00	106.3 $\pm$ 13.7

### *Statistical Analysis*

The effect of player position on game variables was tested in a linear mixed effects model fitted with restricted maximum likelihood, conducted in the lme4 package (Bates *et al.*, 2014) in R version 3.1.1 (Team 2014). P values for coefficients of fixed effects were calculated using Satterthwaite's method of denominator synthesis, conducted in the lmerTest package (Kuznetsova *et al.*, 2013) for R. The response variables were impacts, distance and percentage changes (calculated individually for each player separately and then combined) in the biochemical markers. The fixed predictors in each model were player positions. Player identity was included as crossed random effects to account for the non-independence of marker measures from each player, and the potential for some games to have different effects on all players to others. Although there is no true replication of players (multiple measures are pseudo replicates) and individual levels of these factors cannot be compared, we tested whether there was significant overall variation explained by the random effects using a likelihood ratio test. A residual plot presented any significant outliers that were removed from successive models. For all models, game time and experience were controlled for. All samples were analysed in duplicate and significance was measured as \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ .

### 10.3 Results

Exposure time (minutes played) significantly effects both total distance covered and distance covered at all speed bands as well as the percentage change in urinary total neopterin (NP) ( $p < 0.001$ ). It does not however, have any effect on the percentage change in cortisol or sIgA secretion rate ( $p > 0.05$ ). Experience similarly, has no effect on any variable ( $p > 0.05$ ), while large inter-individual and game variability were observed ( $p < 0.001$ ).

When total distance was analysed from GPS, backs significantly ( $p < 0.01$ ) covered more metres per minute than forwards (Table 10.2). Both inside and outside backs covered a similar distance that was significantly ( $p < 0.05$ ) more than all forward positions (Table 10.1). While there were no differences in metres covered per minute between all positions above 7km/h, backs covered significantly ( $p < 0.01$ ) more distance per minute than forwards above 16, 20 and 25 km/h (Table 10.2). When positions were analysed separately, loose forwards covered more distance than locks and front rowers above 16, 20 and 25 km/h ( $p < 0.01$ ), while inside backs and outside backs covered significantly more distance per minute ( $p < 0.001$ ) than all forward positions (Table 10.2).

The total number of impacts recorded was significantly ( $p < 0.001$ ) higher in forwards than backs (Table 10.3). When impacts were analysed separately, forwards were involved in more tackles, tackle assists ( $p < 0.01$ ) and rucks ( $p < 0.001$ ), but backs tended to carry the ball into contact more regularly ( $p < 0.05$ ). It was found that there were no differences ( $p > 0.05$ ) in the number of total impacts between all forward positions per minute of game time, but significantly more ( $p < 0.001$ ) than both back positions. These however were not significantly different from one another ( $p > 0.05$ ). The types of impacts amongst positions revealed loose forwards complete the highest number of tackles and tackle assists ( $p < 0.05$ ) while outside backs complete the fewest ( $p < 0.01$ ). Meanwhile all forward positions were involved in more rucks per minute of game time than backs ( $p < 0.001$ ). The number of rucks between back positions did not differ, nor did it between forwards (Table 10.3). There was however, significantly more ball carries resulting in an impact by both back positions per minute of game time ( $p < 0.01$ ), while front rowers were involved in the fewest amongst all positions ( $p < 0.05$ ).

**Table 10.2.** Averaged GPS data comparing player positions presented as distance per minute of play. Data is mean  $\pm$  SD.

Position	Total Distance (m)	Distance (>7km/h)	Distance (>16km/h)	Distance (>20km/h)	Distance (>25km/h)
<b>Group</b>	80.5 $\pm$ 17.0	47.8 $\pm$ 14.1	12.5 $\pm$ 6.3	4.4 $\pm$ 3.0	0.71 $\pm$ 1.0
<b>Forwards</b>	77.3 $\pm$ 20.5	45.7 $\pm$ 16.0	9.9 $\pm$ 5.4	2.7 $\pm$ 2.3	0.21 $\pm$ 0.47
<b>Backs</b>	84.7 $\pm$ 10.4**	50.1 $\pm$ 11.1	15.6 $\pm$ 5.9**	6.3 $\pm$ 2.4***	1.3 $\pm$ 1.2***
<b>Front row</b>	71.0 $\pm$ 11.0	41.1 $\pm$ 10.5	7.5 $\pm$ 3.6	1.4 $\pm$ 1.1	0.01 $\pm$ 0.01
<b>Locks</b>	77.4 $\pm$ 7.0	48 $\pm$ 6.8	7.4 $\pm$ 2.2	1.3 $\pm$ 0.8	0.05 $\pm$ 0.01
<b>Loose forwards</b>	86.2 $\pm$ 31.4	51.6 $\pm$ 23.5	15.0 $\pm$ 5.5**	5.3 $\pm$ 2.0***	0.61 $\pm$ 0.66**
<b>Inside backs</b>	86.1 $\pm$ 11.5***	53.8 $\pm$ 10.9**	16.5 $\pm$ 10.8***	5.9 $\pm$ 2.6***	0.75 $\pm$ 0.73***
<b>Outside backs</b>	82.5 $\pm$ 8.4*	44.5 $\pm$ 4.2	14.1 $\pm$ 3.8**	7.1 $\pm$ 2.0***	2.14 $\pm$ 1.20***

Comparisons are made between forwards and backs within each column, and positional groups within each column.

\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

Group (n = 104), Forwards (n = 56), Backs (n = 48)

**Table 10.3.** Averaged video analysis data comparing player positions presented as events per minute. Data is mean  $\pm$  SD.

Position	Total Impacts	Tackles & Tackle Assists	Ball Carries	Rucks
<b>Group</b>	0.47 $\pm$ 0.22	0.13 $\pm$ 0.09	0.09 $\pm$ 0.06	0.24 $\pm$ 0.21
<b>Forwards</b>	0.56 $\pm$ 0.23***	0.15 $\pm$ 0.08**	0.08 $\pm$ 0.05	0.33 $\pm$ 0.25***
<b>Backs</b>	0.36 $\pm$ 0.17	0.11 $\pm$ 0.11	0.11 $\pm$ 0.06*	0.13 $\pm$ 0.09
<b>Front row</b>	0.51 $\pm$ 0.11***	0.14 $\pm$ 0.07	0.06 $\pm$ 0.05	0.32 $\pm$ 0.09***
<b>Locks</b>	0.54 $\pm$ 0.01***	0.16 $\pm$ 0.09	0.10 $\pm$ 0.02*	0.28 $\pm$ 0.06***
<b>Loose forwards</b>	0.64 $\pm$ 0.36***	0.17 $\pm$ 0.09*	0.09 $\pm$ 0.06*	0.37 $\pm$ 0.42***
<b>Inside backs</b>	0.39 $\pm$ 0.20	0.14 $\pm$ 0.12	0.11 $\pm$ 0.07**	0.15 $\pm$ 0.11
<b>Outside backs</b>	0.31 $\pm$ 0.09	0.07 $\pm$ 0.07 <sup>##</sup>	0.12 $\pm$ 0.06**	0.11 $\pm$ 0.03

Comparisons are made between forwards and backs within each column, and positional groups within each column.

\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

<sup>#</sup> Significantly fewer than all other groups

Group (n = 104), Forwards (n = 56), Backs (n = 48)

The change in urinary total NP from pre to post-game for the group, forwards, backs and all positions increased significantly ( $p < 0.001$ ). There was no difference in the percentage change between forwards and backs when controlled for game time ( $p > 0.05$ ). However, even though there was a large range in percentage changes (43.6 – 109.6), the position of lock had a significantly higher percentage increase ( $p < 0.05$ ) than all other positions when game time was controlled (Table 10.4).

**Table 10.4.** A comparison between player positions in changes in urinary total neopterin. The percentage change significance is controlled for game time and all data is presented as mean  $\pm$  SD.

Position	Total Neopterin (nmol/SG <sub>1.020</sub> )		
	Pre-game	Post-game	Percentage Change
<b>Group</b>	4445 $\pm$ 1238	6885 $\pm$ 2295***	61.0 $\pm$ 55.4
<b>Forwards</b>	4847 $\pm$ 1268	7499 $\pm$ 2422***	61.2 $\pm$ 58.4
<b>Backs</b>	3934 $\pm$ 1024	6214 $\pm$ 1870***	60.7 $\pm$ 52.4
<b>Front row</b>	4647 $\pm$ 1238	6390 $\pm$ 2064***	43.6 $\pm$ 46.8
<b>Locks</b>	5159 $\pm$ 1625	10868 $\pm$ 2080***	109.6 $\pm$ 83.1*
<b>Loose forwards</b>	4883 $\pm$ 1236	7304 $\pm$ 1570***	57.5 $\pm$ 38.2
<b>Inside backs</b>	3834 $\pm$ 1008	5790 $\pm$ 1512***	55.3 $\pm$ 58.2
<b>Outside backs</b>	4107 $\pm$ 1122	7140 $\pm$ 2342***	71.6 $\pm$ 38.3

Pre-game is compared to post-game, while the percentage changes are compared to one another.

\*  $p < 0.05$ , \*\*\*  $p < 0.001$

Group (n = 104), Forwards (n = 56), Backs (n = 48)

Similar observations were seen for changes in salivary cortisol with post-game concentrations significantly higher ( $p < 0.001$ ) than pre-game for all positions (Table 10.5). There was no difference between forwards and backs, while locks were the only position that had a significantly higher cortisol percentage change when controlled for game time ( $p < 0.05$ ).



There was also a large inter-individual variation ( $p < 0.001$ ) which resulted in large error values and range in percentage (170.8 – 277.7).

**Table 10.5.** A comparison between player positions in changes in salivary cortisol. The percentage change significance is controlled for game time and all data is presented as mean  $\pm$  SD.

Position	Pre-game	Cortisol (nmol/L)	
		Post-game	Percentage Change
<b>Group</b>	17.7 $\pm$ 8.1	44.0 $\pm$ 21.9***	194.6 $\pm$ 189.0
<b>Forwards</b>	17.4 $\pm$ 8.7	44.2 $\pm$ 23.4***	195.8 $\pm$ 187.4
<b>Backs</b>	18.0 $\pm$ 7.5	44.2 $\pm$ 20.4***	193.1 $\pm$ 193.8
<b>Front row</b>	17.5 $\pm$ 8.5	37.9 $\pm$ 19.1***	172.2 $\pm$ 209.3
<b>Locks</b>	14.7 $\pm$ 3.5	58.0 $\pm$ 13.1***	277.7 $\pm$ 135.9*
<b>Loose forwards</b>	19.6 $\pm$ 11.0	46.2 $\pm$ 22.8***	180.9 $\pm$ 172.9
<b>Inside backs</b>	17.1 $\pm$ 7.1	43.1 $\pm$ 22.3***	204.3 $\pm$ 208.9
<b>Outside backs</b>	19.9 $\pm$ 7.8	45.1 $\pm$ 16.3***	170.8 $\pm$ 165.8

Pre-game is compared to post-game, while the percentage changes are compared to one another.

\*  $p < 0.05$ , \*\*\*  $p < 0.001$

Group (n = 104), Forwards (n = 56), Backs (n = 48)

Salivary IgA for the group was observed to decrease from pre to post-game (Table 10.6). When analysed separately, both forwards and backs showed a significant decrease, however there was no significant difference between the two when corrected for game time ( $p > 0.05$ ). Between positions however, players in the front row and loose forwards were the only positions that showed a significant decrease, with outside backs having a significantly higher percentage change than all other positions ( $p < 0.05$ ).

**Table 10.6.** A comparison between player positions in changes in sIgA secretion rate. The percentage change significance is controlled for game time and all data is presented as mean  $\pm$  SD.

Position	Pre-game	sIgA ( $\mu\text{g}/\text{min}$ )	
		Post-game	Percentage Change
<b>Group</b>	401.3 $\pm$ 254.4	298.3 $\pm$ 148.1***	- 1.9 $\pm$ 70.3
<b>Forwards</b>	442.0 $\pm$ 272.3	308.0 $\pm$ 159.1***	- 11.8 $\pm$ 60.7
<b>Backs</b>	370.6 $\pm$ 218.7	285.5 $\pm$ 133.2*	10.6 $\pm$ 79.9
<b>Front row</b>	498.7 $\pm$ 353.3	303.1 $\pm$ 169.1**	- 26.6 $\pm$ 47.8*
<b>Locks</b>	389.0 $\pm$ 195.5	369.5 $\pm$ 161.1	21.3 $\pm$ 64.3
<b>Loose forwards</b>	392.9 $\pm$ 146.6	272.2 $\pm$ 148.1*	- 8.8 $\pm$ 72.9*
<b>Inside backs</b>	360.5 $\pm$ 235.4	294.4 $\pm$ 139.0	14.5 $\pm$ 79.6
<b>Outside backs</b>	330.3 $\pm$ 155.9	267.1 $\pm$ 124.6	2.8 $\pm$ 83.5*

Pre-game is compared to post-game, while the percentage changes are compared to one another.

\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

Group ( $n = 104$ ), Forwards ( $n = 56$ ), Backs ( $n = 48$ )

## 10.4 Discussion

This study provides evidence that the contemporary demands (impacts and distance) of professional rugby are position dependent as previously noted (Deutsch *et al.*, 2007), while changes in psychophysiological stress are independent. The presentation of data as events or distance per minute makes it more precise and informative about the demands of the game, but difficult to compare to previous research (Cunniffe *et al.*, 2009; Deutsch *et al.*, 1998; Quarrie and Hopkins 2007).

All positions in rugby union have specific requirements with players chosen based on their endurance, speed, agility, power, flexibility and sport-specific skill (Duthie *et al.*, 2003). As a result of rule changes however, the increased speed of play has changed the positional demands (Hughes and Blunt 1998). Based on the evidence presented, forwards complete more total impacts, tackles and tackle assists, as well as rucks on offence and defence which requires substantial muscular strength (Reilly 1997) as a result of their proximity to the contest and similar to that previously shown (Quarrie *et al.*, 2013). The larger body type and physical characteristics of these players allows them to complete their tasks of securing

possession and halting the progression of the opposition. When analysed separately, it suggests no one position completes more “work” than the other in normal game play, even though they each have specific jobs they have to complete requiring large amounts of strength and power such as scrums (Quarrie and Wilson 2000). However, careful consideration of the evidence shows a trend toward loose forwards being involved in the highest number of events per minute of game time. Backs meanwhile, because of their smaller stature (Table 10.1) and ability to run at higher speeds, are more involved in carrying the ball and advancing their team over the advantage line through speed and skill which is in contrast to previous research (Quarrie *et al.*, 2013); a difference most likely attributed to the difference in competition level and/or the difference in data presentation. A lack of difference between inside and outside backs is similar to that observed between forwards; however as a result of the positional play required of outside backs, their total number of tackles or tackle assists is significantly smaller.

Furthermore, backs cover significantly more distance than forwards which is in accordance with previous work (Roberts *et al.*, 2008; Suárez-Arrones *et al.*, 2012). Additionally, backs also cover more distance at high speeds which is similar to that previously noted (Austin *et al.*, 2011; Deutsch *et al.*, 1998; Maud 1983) while the percentage of time spent at low intensity is similar to that shown by Deutsch *et al.* (Deutsch *et al.*, 1998). Inside backs however, tend to cover the greatest distance per minute of game time which is likely a result of the greater ball-carrying events and positional play on offence and defence which is still in accordance with rugby in the 1980’s (Docherty *et al.*, 1988). The significantly less distance covered at the high speeds in the front row and locks may be a result of their positional requirements preventing them from attaining high speed from breakdown to breakdown, or their inability to reach these speeds due to physiological restrictions (Table 10.1). When combining the two pieces of data that are traditionally used to gauge the stress of a player, loose forwards tend to complete the greatest workload which would suggest it is one of the fittest and physically demanding positions and similar to that previously shown (Lacome *et al.*, 2014). This type of information can therefore be used to adapt and change specific training requirements, which can also be utilized for other positions. Forwards as an example would benefit most from muscle hypertrophy, strength and power to prepare their body for the rigors of high force and frequency collisions (Usman *et al.*, 2011). As a result of professionalism, each player is trained specifically for a purpose, and it is this information

that backs up training methods that optimizes each player for repeatable and optimal performances.

The significant change in salivary cortisol, salivary immunoglobulin A (sIgA) and urinary total NP is in accordance with previous studies in rugby union (Cunniffe *et al.*, 2010; Lindsay *et al.*, 2014b). It identifies professional rugby as a sport of severe intensity causing changes in several key physiological systems. Salivary immunoglobulin A has been routinely utilized in several exercise contexts (Southworth *et al.*, 2013). The significant decrease provides evidence of immune system suppression and an increased susceptibility to infection post-game. Similarly, cortisol's large post-game increase is indicative of both physiological and psychological stress changes that can also cause alteration to immune system function of immune system function (Shinkai *et al.*, 1996). Total NP meanwhile, provides a complete understanding of the inflammatory processes involved following a game. Its release following rugby union is governed through  $\gamma$ -interferon stimulation of macrophages which become activated following the structural stress associated with the physicality of the game.

The difference between forwards, backs and positions however is mostly negligible across all three markers (Tables 10.4 - 6) except for the higher response of locks to NP and cortisol and larger decrease of the front row, loose forwards and outside backs for sIgA. As stated, backs typically cover more distance and are involved in less impacts while forwards are involved in more impacts and cover less distance which all seems to correspond to a similar stress outcome. This indicates these markers are not only measuring metabolic stress or emotional stress, but potentially a combination of both. It is also highly likely that unmeasured factors such as impact force and the individual physiology of each player (aerobic and anaerobic thresholds) dominate the change in biomarker response. This is evident from the large inter-individual variations of percentage change (Tables 10.4 – 6) that indicates every individual player should be monitored as their own entity.

### *Conclusion*

These types of analyses can be used to both manage and monitor players on an individual positional basis to adapt training around the requirements of each position. It is evident individual physiological differences and unmeasured factors are responsible for changes in psychophysiological stress across positions, however forwards are involved in more impacts and cover fewer metres than backs per minute of game time.

# Chapter 11

## Effect of Varied Recovery Interventions on Markers of Psychophysiological Stress in Professional Rugby Union

### Abstract

Rugby union is a physical demanding sport that requires optimum recovery between games to maintain performance levels. Analysis of four unique biochemical markers of stress is measured here to determine which recovery strategy currently in use by a professional team provides the necessary requirements for sustained performance. Urine and saliva samples were collected from 37 professional rugby players before, immediately after and 36 hours after five home games and analysed by ELISA and HPLC for urinary myoglobin, total neopterin (neopterin + 7,8-dihydroneopterin), salivary cortisol and immunoglobulin A. Subjects completed a CWI or pool session, donned compression garments, consumed protein and carbohydrate food and fluid, and slept for eight hours post-game. The following day subjects choose from one or a combination of CWI, pool session or active recovery/stretching to complete. There was no difference between the recovery protocols for cortisol, total neopterin, immunoglobulin A concentration or myoglobin at 36 hours post-game. Immunoglobulin A secretion rate significantly increased above pre-game levels at 36 hours post-game for all protocols, however protocol three did not increase as much ( $p = 0.038$ ). Total neopterin was also significantly increased above pre-game levels at 36 hours post-game for all protocols. This study provides evidence that the immediate post-game recovery intervention following a game of professional rugby union may be the most important aspect of psychophysiological player recovery, irrespective of the “next-day” intervention. The concentrations of total neopterin and immunoglobulin A suggest these professional rugby players are still in a state of recovery 36 hours post-game.

### 11.1 Introduction

Rugby is a contact sport that incorporates periods of high intensity anaerobic exercise interspersed with low intensity aerobic exercise and high force impacts (Cunniffe *et al.*, 2009; Pain *et al.*, 2008). Players have adapted at the onset of the professional era becoming faster, stronger, bigger and more skilful across all positions (Fuller *et al.*, 2012) which has led to an

increase in rucks, tackles, work to rest ratios and tackle forces due to the velocity of collision (Austin *et al.*, 2011; Quarrie and Hopkins 2007; Usman *et al.*, 2011)

Competing in arguably the most physically demanding sport in the world for approximately 40 weeks/year requires recovery interventions that manage players physiologically and psychologically to maintain performance without running the risk of over-reaching or over-training. Monitoring player recovery is traditionally achieved through questionnaires of perceived fatigue and soreness (Johnston *et al.*, 2013), performance tests (Rowell *et al.*, 2009), neuromuscular function (Twist *et al.*, 2012) and blood and salivary markers (McLellan *et al.*, 2011a). While all have provided some means of assessing over-training, biochemical analysis provides factual, quantitative and individualised information about the health status and recovery of athletes.

Neopterin, cortisol, salivary immunoglobulin A (sIgA) and myoglobin are unique biomarkers that have all been shown to change in response to exercise and competitive rugby (Cunniffe *et al.*, 2010; Lindsay *et al.*, 2014b). Neopterin is a marker of immune system activation and ratio indicator of oxidative stress that has been used to diagnose and monitor infection and exercise inflammation (Lindsay *et al.*, 2014b; Wachter 1992). In conjunction with myoglobin, a protein found specifically in skeletal muscle responsible for O<sub>2</sub> transport and used to quantify muscle damage severity in both the clinical and exercise fields (Feinfeld *et al.*, 1992; Takarada 2003), they can provide a snapshot into exercise induced physiological stress to observe athlete recovery progression. Similarly, salivary cortisol has been used multiple times to assess exercise induced stress (Cunniffe *et al.*, 2010) which provides information about the psychophysiological profile of an athlete during both acute and chronic training/competition. Together with sIgA, the most commonly utilized biomarker for assessing immune system status following exercise (Gleeson *et al.*, 1999), can also provide useful information about psychophysiological recovery of athletes.

Although there is a great deal of research available about the usefulness of recovery strategies on different facets of exercise and sport, the research still remains equivocal and difficult to grasp. “Stress” is an important aspect that forces adaptation and improves an individual’s performance. However, when rugby union players are expected to compete in a highly intense game and return to the gym and training sessions ~36 hours later, their recovery is especially important to avoid over-training and optimise performance. Recovery strategies

currently include active recovery (King and Duffield 2009), stretching (Egan *et al.*, 2006), hydration/nutrition (Berardi *et al.*, 2006), sleep (Oliver *et al.*, 2009), massage and physiotherapy (Weerapong and Kolt 2005), compression garments (Hamlin *et al.*, 2012; Kraemer *et al.*, 2001a), cold water immersion and contrast water therapy (Hamlin 2007; Vaile *et al.*, 2008). Some have proven more effective than others, but there is little research on the effect of active recovery in a pool, the use of a combination of strategies, and research investigating the real-time effects of recovery interventions in current practice by professional sports teams during a competition (Gill *et al.*, 2006).

Therefore, the aim of this study was to examine the effectiveness of the selected biomarkers in their prediction of player recovery, and determine which of the current recovery strategies employed by a professional rugby union team was the most efficient at returning players to a psychophysiological state that allows for resumption of normal training 36 hours post-game.

## 11.2 Methods

### *Subjects*

Thirty-seven professional rugby players from a Super 15 franchise volunteered for the study. Of the 37 who volunteered, 23 were selected to play each game with 20 providing samples at all three time points in game one (eight forwards, 12 backs), 16 in game two (nine forwards, seven backs), 14 in game three (seven forwards, seven backs), 17 in game four (ten forwards, 7 backs) and 14 in game five (eight forwards, six backs). Subjects had to play at least 30 minutes before being included.

Details of subjects enrolled in the study are shown in Table 11.1. The experimental protocol was approved by the University of Canterbury Human Ethics Committee (Christchurch, New Zealand) and all subjects were informed (Appendix M) of the risks involved in the study before their written consent was obtained (Appendix L).

### *Experimental Protocol and Sample Collection*

Urine and saliva samples were collected pre-game (within 120mins), post-game (within 60mins) and 36 hours post-game ( $\pm 2$  hours) for five home games which was in accordance with subject access permitted by team management. Post-game samples were collected before any subject had completed any of their post-game recovery intervention.

*Urine and saliva sample collection*

Refer to Materials and Methods (Chapter 2.2)

**Table 12.1.** Subject characteristics. Data is mean  $\pm$  SD.

Age (years)	26.0 $\pm$ 3.5
Height (m)	1.86 $\pm$ 0.07
Weight (kg)	104.5 $\pm$ 9.3

*Recovery Protocols*

Immediately following each game, subjects were given one 750 mL bottle of H<sub>2</sub>O, one protein shake (whey protein, berries, honey, yoghurt and banana), one of either coconut milk or Powerade<sup>®</sup> and advised to graze at a food table that included wedges, fish bites, salmon sashimi, fruit platter and a mixture of tuna, salmon and chicken sushi. Each subject was advised to consume the same approximate amount of food and liquid following each game. After game one, subjects completed a pool session (described below) followed by donning lower body compression garments until the next morning and eight hours of sleep, while after games two to five they were instructed to complete a CWI (described below) followed by the use of lower body compression garments until the next morning and eight hours of sleep. The following day, each subject had three protocols to choose from. They could do one, a mixture, or all three.

**CWI. Cold water immersion** – Participants immersed their bodies up to the thoracic/cervical vertebrae juncture in a temperature controlled bath (8-12°C) for ten minutes.

**PS. Pool session** – Participants completed a pool session in an Olympic sized pool (50m) that included ten lengths dynamic activity (jogging / skips / side skip / high knees / lunges / walking quad and glute stretch ) followed by ten lengths of any stroke.



AS. **Active/Stretching** – Participants completed 20 minutes of either light cycling or power walking followed by 10mins of stretching with a band (calves / quads / hamstrings / groin / lower back / hip flexors / glutes).

When samples were collected 36 hours post-game, each subject was asked to fill out a questionnaire stating what protocols they had completed. Subjects were then pooled into four groups based on their recovery protocol they completed (Table 12.2.).

**Table 11.2.** Recovery protocols. PCA (N = 20), CCPA (N = 17), CPA (N = 30), CA (N = 13).

Group	Post-game	Day after
PCA	PS + compression + 8hrs Sleep	CWI +/- AS
CCPA	CWI + compression + 8hrs Sleep	CWI + PS +/- AS
CPA	CWI + compression + 8hrs Sleep	PS +/- AS
CA	CWI + compression + 8hrs Sleep	AS or nothing

### *Sample Analysis*

Refer to Materials and Methods (Chapter 2.2)

### *Statistical Analysis*

The effect of recovery intervention on the change in concentrations of each marker was tested in a linear mixed effects model adjusted for restricted maximum likelihood, conducted in the lme4 package (Bates *et al.*, 2014) in R version 3.1.1 (Team 2014). P values for coefficients of fixed effects were calculated using Satterthwaite's method of denominator synthesis, conducted in the lmerTest package (Kuznetsova *et al.*, 2013) for R. Each marker was analysed as the response variable in a separate model. The fixed predictor in each model was time (pre vs. post vs. 36 hours post-game). Player identity was included as crossed random effects to account for the non-independence of marker measures from each player. As there

is no true replication of players (multiple measures are pseudo replicates), individual levels of this factor could not be compared, so we tested whether there was significant overall variation explained by the random effects using a likelihood ratio test.

### 11.3 Results

The rugby matches examined in this study caused a 57% increase in salivary cortisol ( $p < 0.001$ ), 64% increase in urinary total neopterin (NP) ( $p < 0.001$ ), 2927% increase in myoglobin ( $p < 0.05$ ), 18.5% decrease in sIgA concentration ( $p < 0.01$ ) and 26.7% decrease in secretion rate ( $p < 0.01$ ) (Figures 11.1 - 5).

There was no difference in the 36 hours post-game myoglobin concentration between protocols compared to pre-game, nor was there a difference in the concentration at 36 hours for each protocol (Fig. 11.1). The variation explained by the subjects was not significant; however the post-game concentrations did significantly differ between protocols ( $p < 0.05$ ).

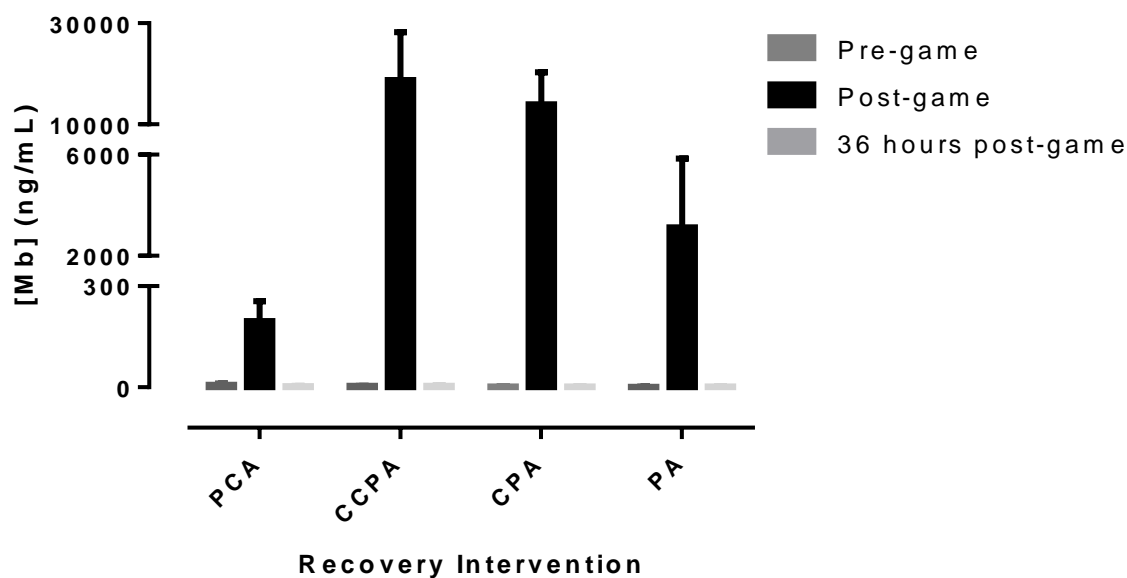
sIgA concentration (Fig. 11.2) was significantly higher at 36 hours post-game compared to pre-game for all recovery protocols ( $p < 0.001$ ). For CCPA, CPA and CA, the increase was not as great compared to PCA and significantly so for CPA ( $p = 0.038$ ). There was also no difference between the pre-game and post-game concentrations for all protocols ( $p > 0.1$ ), however the variation between the subjects response to the game and recovery protocol was significantly different ( $p < 0.001$ ).

Similarly, sIgA secretion rate (Fig. 11.3) was significantly increased at 36 hours post-game compared to pre-game for all recovery protocols ( $p < 0.01$ ) with significant differences observed between the subjects response ( $p < 0.001$ ). CPA had a significantly lower 36 hour concentration than all other protocols ( $p = 0.019$ ), however the post-game concentration of this protocol was significantly lower as well ( $p = 0.004$ ).

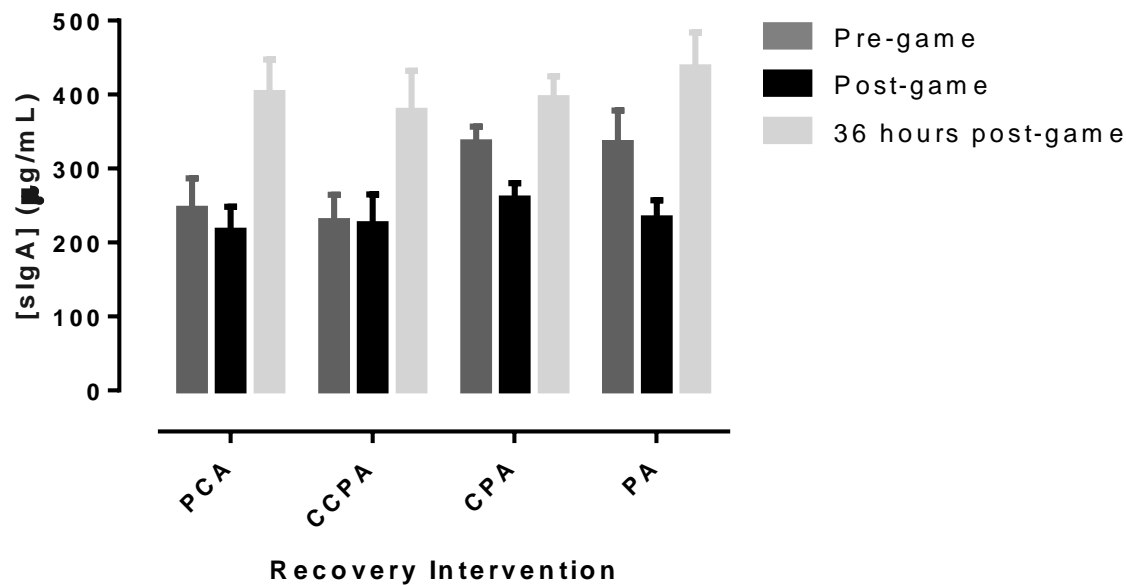
For all recovery interventions, 36 hours salivary cortisol (Fig. 11.4) was not significantly different from pre-game concentrations, nor was there a difference in the 36 hours concentrations or variation between players. There was however, a significant difference in the post-game concentration between protocols ( $p < 0.05$ ).

For all recovery protocols, total NP (Fig. 11.5) significantly increased from pre to post-game and significantly decreased from post-game to 36 hours post-game ( $p < 0.05$ ). Concentrations at 36 hours however, were significantly higher than pre-game concentrations

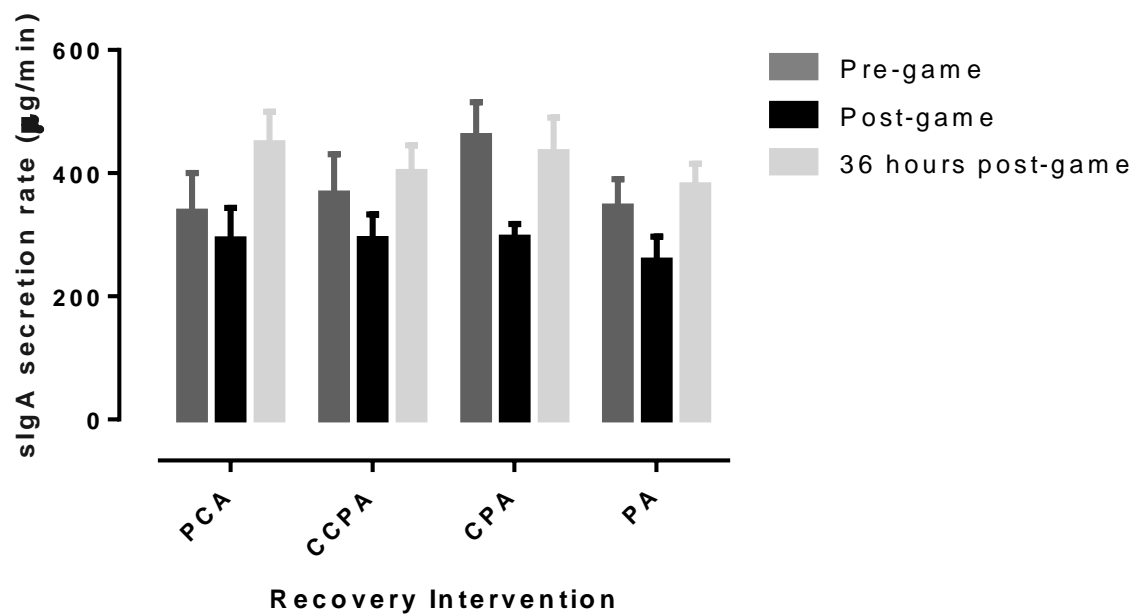
for all protocols ( $p < 0.05$ ). There was also no difference between protocols at any time point, however the response between each subject to the game and protocol was significantly different ( $p < 0.001$ ).



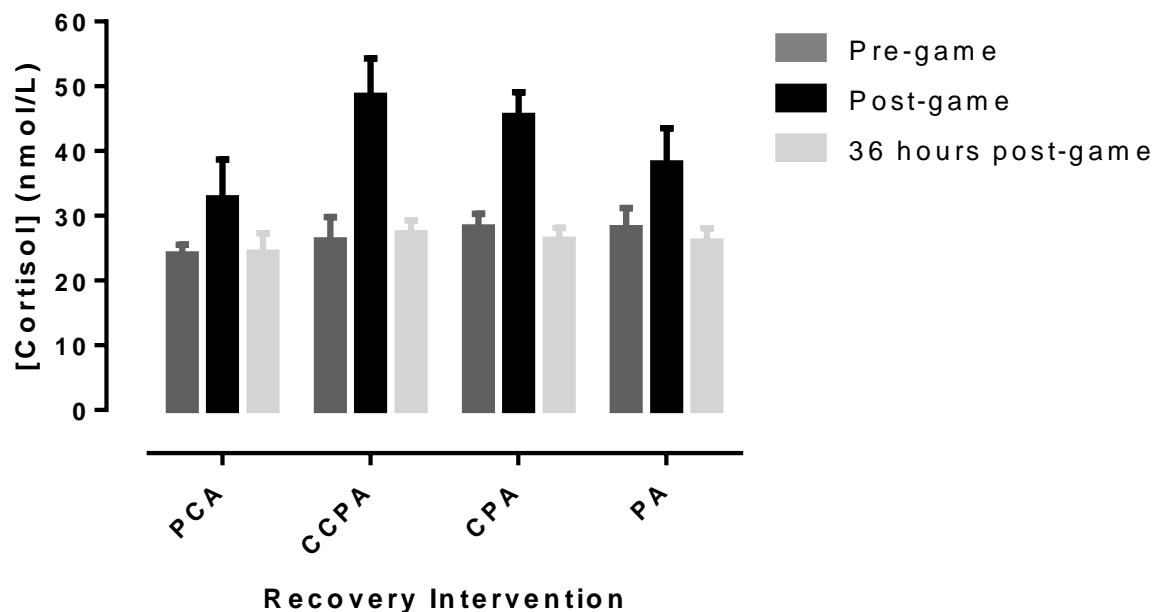
**Figure 11.1.** Urinary myoglobin concentration changes following each recovery intervention. Data is presented as mean  $\pm$  SD.



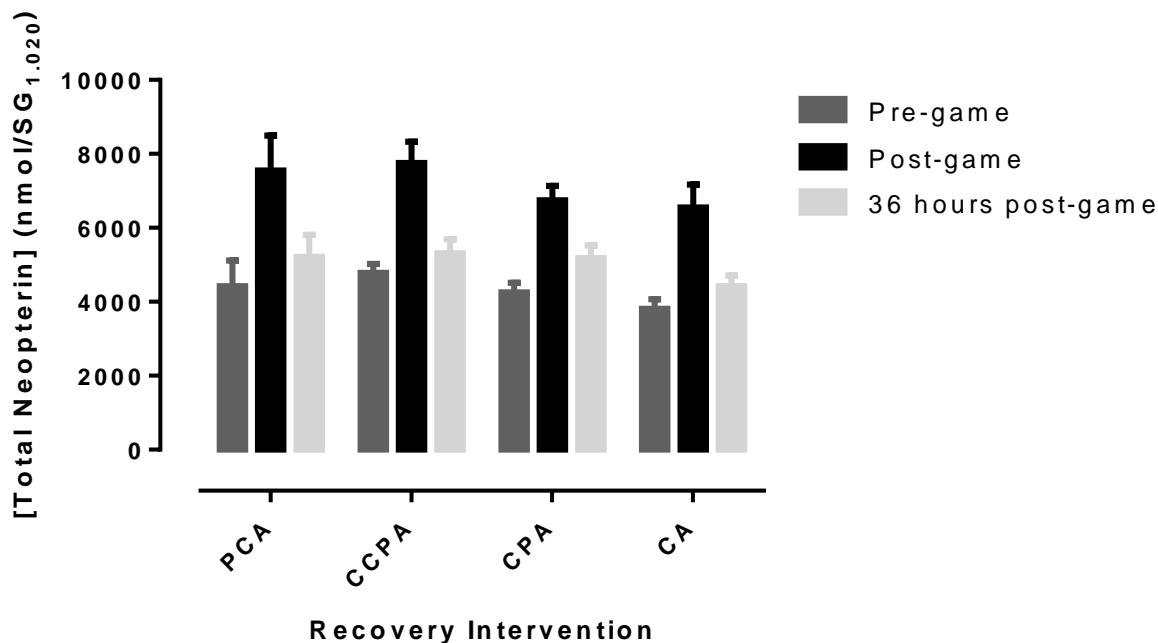
**Figure 11.2.** sIgA concentration changes following each recovery intervention. Data is presented as mean ± SD.



**Figure 11.3.** sIgA secretion rate changes following each recovery intervention. Data is presented as mean ± SD.



**Figure 11.4.** Salivary cortisol changes following each recovery intervention. Data is presented as mean  $\pm$  SD.



**Figure 11.5.** Urinary total NP changes following each recovery intervention. Data is presented as mean  $\pm$  SD.

### 11.4 Discussion

Each of the markers utilized in this study demonstrates a unique non-overlapping representation of stress that together can provide an adequate understanding of the psychophysiological response of an individual's recovery. The results of the present study confirm previous research illustrating rugby union induced physiological stress (Cunniffe *et al.*, 2010; Gill *et al.*, 2006; Lindsay *et al.*, 2014b). Specifically, a game of rugby union results in relatively immediate increases in post-game concentrations of cortisol, total NP, myoglobin and a decrease in sIgA (Cunniffe *et al.*, 2010; Takarada 2003). It also suggests the immediate post-game recovery intervention utilized in this study may be the most important aspect in psychophysiological player recovery; however this cannot be definitively confirmed because there were no players who did not complete the post-game intervention. This is accomplished through hydrostatic pressure (Wilcock *et al.*, 2006) and vasoconstriction that aids in metabolite removal, a reduction in swelling and muscle soreness (Cochrane 2004), increase in protein synthesis, and restoration of the immune, endocrine and nervous systems (Frank 2006; Howarth *et al.*, 2009; Swenson *et al.*, 1996).

The individual difference among the subject's response to the game and protocol provides evidence of variation. It suggests there is not only a difference in the level of stress to the games, but the response to the protocol is also individualised as well. What this means for recovery protocols in a group situation is unclear, however one protocol may not be suitable for all players, nor can global positioning system (GPS) or video analysis data be used precisely to predict what type of recovery protocol an individual player might benefit most from. The results of this research suggest as long as a player completes the immediate post-game recovery intervention of this study, they have a high probability of returning to a psychophysiological state that allows for a return to training in a professional rugby team.

Muscle damage has been shown to cause a decrease in performance (Twist and Eston 2005). Effect of recovery interventions on exercise induced muscle damage has been repeatedly assessed (Gill *et al.*, 2006). The significant elevation above pre-game levels is in accordance with previous research (Takarada 2003) and highlights the extent of muscle damage following games of rugby union. Its return to pre-game levels ( $< 5$  ng/ml) following all four recovery protocols (Fig. 12.1) suggests the muscle integrity is no longer compromised as a result of the recovery processes involved. This result is in contrast to previous research that

has shown creatine kinase elevated up to 84 hours into recovery (Gill *et al.*, 2006). Similar protocols were in place, but the difference in muscle damage marker concentration may be related to the marker itself, the type of analyses conducted, or the level of damage associated with the games. Of note however, was the difference in post-game concentrations between the different protocols. Whether PCA and CA were capable of returning substantially higher concentrations to pre-game levels at 36 hours cannot be ascertained, which therefore suggests the integrity of the muscle cell membrane is restored using any of the four protocols.

Salivary IgA concentration and secretion rate have been used to assess immune system function in several acute and chronic exercise protocols (Gleeson *et al.*, 1999). The immediate post-game suppression suggests rugby union is of a severe intensity which is in accordance with previous high intensity exercise (Nieman *et al.*, 2002) but in contrast to a rugby specific study (Koch *et al.*, 2007). This is most likely attributed to the level of competition with the latter examining collegiate players. However, the significant increase above pre-game and post-game concentrations at 36 hours for all protocols (Fig. 12.2 and 12.3) is in accordance with previous work in competitive cyclists who showed a significant decline in sIgA secretion rate immediately post but returned to pre-exercise levels by 24 hours (Mackinnon *et al.*, 1989). This is suggestive of a rebound effect to the significant elevations in muscle damage, stress and inflammation sustained during the game, while sIgA change remains independent of recovery protocol. Players in the CPA group however, did not elevate sIgA concentration and secretion rate to the same extent of the others. This may be explained by the lower post-game concentration observed for those subjects who completed CPA, and which ties in with the higher cortisol and higher myoglobin concentrations observed as well. Over activation may provide evidence of delayed recovery; however it does provide useful information in regard to the “open-window” concept, a phenomenon shown to occur following intense exercise that leaves a person susceptible to clinical and subclinical infection (Nieman 1994). Normally considered to last several hours post-exercise, this is the first evidence that in a sport dominated by high force and frequency impacts, a player’s immune system is not in a compromised state when resumption of normal training is restarted.

The post-game cortisol response is similar to that previously reported (Lindsay *et al.*, 2014b). As both a measure of physiological and psychological stress, cortisol is ideally suited to establish the overall health of an athlete, but which can also be influenced by non-exercise related stress (Strahler *et al.*, 2010b). The results indicate cortisol returns to pre-game levels 36 hours post-game irrespective of the recovery protocol which has been previously noted (Lindsay *et al.*, 2014b). This is likely achieved through a reduction in swelling and inflammation and a mental break. Similar to the myoglobin response, cortisol was significantly higher in those players who did recovery protocol two and three (Fig. 11.4). These results cannot determine whether recovery PCA and CA would have been capable of returning levels to pre-game, but suggest those who completed these protocols may have recognized they were in better shape post-game and therefore did not feel another CWI or pool session the following day was required.

In a sport that causes severe muscle damage (Smart *et al.*, 2008), the acute phase inflammatory response is an essential tool for monitoring recovery in sport. The immediate increase in total NP post-game (Fig. 11.5) provides evidence of macrophage activation at sites of soft tissue damage which is likely in response to the impact induced muscle damage. The significant decrease in total NP at 36 hours post-game in comparison to post-game identifies the effectiveness and similarity of each recovery protocol, however the significant increase above pre-game concentrations suggests the subjects have not completely recovered from the stress of the game; an important piece of evidence that can be used to manage training load and recovery to maximize performance. This is similar to other markers investigating inflammation in rugby union that have shown C-reactive protein (CRP) and interleukin-6 (IL-6) elevation 14-38 hours post-game (Cunniffe *et al.*, 2010). These results do however show that there is no difference in the inflammatory response 36 hours post-game between different protocols.

### *Conclusion*

The overall results of this research suggest the immediate post-game recovery intervention is the main influential factor on the 36 hour post-game response of physiological and psychological stress markers in rugby union. This may provide useful information for future use when planning recovery strategies in a professional rugby team, however it would seem those players who experienced severe stress intuitively choose to complete the most intense



recovery protocol (CCPA). It would also seem there is an over activation of the immune and inflammatory system 36 hours post-game which is more than likely related to an adaptation effect. Whether this timeline can be shortened by the use of more “intense” protocols that could include massage or contrast water therapy is something that could be explored further.



# Study 5

Involves two investigations that builds on the previous evidence contained in Studies one to four. The first involves the development of a simple, effective and repeatable reverse-phase chromatography assay for the quantification of urinary myoglobin whilst investigating stabilisation techniques that often plague its precise measurement. The second investigation measures time course changes of selected biomarkers using the newly developed method following a mixed martial arts contest and the effect of cold water immersion.

# Chapter 12

## Urinary Myoglobin Quantification by HPLC; an Alternative measurement for Exercise-Induced Muscle Damage

### Abstract

Urinary myoglobin quantification is currently limited by certain assay and sample storage restrictions. This study investigates a means of quantifying urinary myoglobin through the development of a novel RP-HPLC method which is both simple and non-invasive. It also investigates the effect of storage and alkalization on the stability of urinary myoglobin. Myoglobin was analysed using a RP-HPLC method that was validated by precision and repeatability experiments. To further corroborate these findings, urinary myoglobin was collected from an elite mixed martial artist before and after a contest. Myoglobin stability was determined through spiked urine samples (10, 100 and 1000  $\mu\text{g/mL}$ ) stored at various temperatures over an eight week period using alkalization and dilution in a pH 7 buffer to inform sample storage protocols. The RP-HPLC method produced linearity from 5 – 1000  $\mu\text{g/mL}$  ( $R^2 = 0.997$ ) with intra- and inter-assay CV's ranging from 0.32% to 2.94%. Recovery from spiked samples ranged from 96.4 – 102.5%, while myoglobin remained stable at 4°C when diluted in 10mM ammonium acetate pH 7 buffer after 20 hours. Storage length and conditions were shown to have significant effects ( $p < 0.05$ ) on myoglobin stability in urine. A lower sample storage temperature ( $p < 0.01$ ) and a greater initial myoglobin concentration attenuated the degradation process ( $p < 0.01$ ). Significant increases in urinary myoglobin ( $p < 0.05$ ) were observed following the mixed martial arts contest with concentrations returning to pre-contest levels 24 hours later. The RP-HPLC method described provides a simple and repeatable assay for the quantification of urinary myoglobin. This method, which is both quantitative and non-invasive, could be used to inform the severity of crush injury, presence of myocardial infarction and rhabdomyolysis, and for assessing physiological stress of athletes through its simplicity and speed of analysis.

### 12.1 Introduction

Myoglobin is a key marker of muscle damage. It has notably been employed to assess the severity of injury and monitor the recovery of individuals suffering from rhabdomyolysis

(Alterman *et al.*, 2007), myocardial infarction (Powell *et al.*, 1984) and accidental trauma (Genthon and Wilcox 2014). Muscle damage quantification is also pertinent to the diagnosis of exercise-induced physiological stress for the management of athlete recovery and performance. To this end, myoglobin has been routinely measured in several exercise protocols ranging from rugby union (Lindsay *et al.*, 2014b) to ultra-endurance exercise (Wallberg *et al.*, 2011). The methodology employed for this measurement ranges from sensitive and expensive immunoassays, dipsticks, histo-chemical staining, radio-immunoassays (RIA), and the relatively inexpensive liquid chromatograph assays (Feinfeld *et al.*, 1992; Lindsay *et al.*, 2014b; Maerker-Alzer *et al.*, 1986; Royds *et al.*, 1985).

Myoglobin, the hemoprotein that facilitates oxygen storage for sustained oxidative phosphorylation in muscle fibres has limited assay availability and significant concerns surrounding its stability in urine (Chen-Levy *et al.*, 2005). Enzyme linked immunosorbent assays (ELISA) are capable of quantifying myoglobin concentrations through their rapid and sensitive processing time. de Waard *et al.* (de Waard and van't Sant 2009) however have highlighted their instability and inaccuracy suggesting that all immunoassay-based myoglobin assays are unsuitable. Similarly, urinary dipsticks are unable to differentiate clearly between myoglobin and haemoglobin and do not offer a quantification method (Anigilaje and Adedoyin 2013). When concentrations range from 3.2 to 3000 mg/L following serious trauma, myocardial infarction and professional rugby (Lindsay *et al.*, 2015b; Lindsay *et al.*, 2015c; Powell *et al.*, 1984; Smith 1968), repeated handling and the inability to specifically quantify muscle damage severity leaves immunoassay options rather impractical. Likewise, compounding effects such as pH, temperature, freezing and unidentified urinary compounds smaller than 10 kDa routinely affect the ability to accurately quantify myoglobin (Chen-Levy *et al.*, 2005). It is suggested samples are pH adjusted upon collection to approximately 7 – 8.5 and analysed immediately to prevent degradation (de Waard and van't Sant 2009). In conjunction with the impracticality of immediate analysis and the extended high performance liquid chromatography (HPLC) assay time, stability of urinary myoglobin is essential.

The need for a reliable, repeatable, simple and cost-effective assay for the quantification of muscle damage through urinary myoglobin following both physical exercise and serious trauma is essential. Several size-exclusion, anion exchange and RP-HPLC techniques have

been utilized for protein and myoglobin determination (Liang *et al.*, 2001; Powell *et al.*, 1984; Sviridov *et al.*, 2006), however they are somewhat outdated, co-elute proteins of similar size, un-validated in urine or require more than one preparative process. RP-HPLC methods commonly utilize water and an organic solvent for separation and identification which have shown a recovery of ~ 100 % following addition to an extract (Oellingrath *et al.*, 1990). We have adopted this methodology to develop, validate and report a similarly reliable RP-HPLC method that quantifies myoglobin in urine by simple UV detection that negates the use of invasive procedures. Whilst HPLC with chemiluminescence detection has a sensitivity limit of 10 ng/mL in human serum (Maltsev *et al.*, 1987), and immunoassays have a maximum detection limit of 1 µg/mL, they are not particularly useful or clinically relevant where serious trauma arises.

The aims of this research are to develop a simple and non-invasive RP-HPLC method to reliably quantify urinary myoglobin following traumatic stress, and to investigate the effect of alkalization and sample dilution on urinary myoglobin stability.

## 12.2 Methods

### *Precision Studies*

Spectral analyses of commercially supplied pure human (Abcam, ab96036) and equine (Sigma Aldrich, M0630) skeletal myoglobin were conducted to identify the optimum absorbance level of myoglobin, while spectral analysis at both 210, 280 and 400 nm were conducted by RP-HPLC on spiked myoglobin urine samples to identify any interference from other urinary compounds. Intra-assay precision was evaluated using 20 replicates of a single urine sample in a single analytical run spiked with 10, 100 and 1000 µg/mL myoglobin standard. Inter-assay precision was evaluated using 20 replicates of a single urine sample on four consecutive days for each of the three concentrations while a calibration curve was established using myoglobin standards ranging from 5 – 1000 µg/mL for both human and equine skeletal myoglobin.

### *Recovery and Stability*

A urine sample was spiked with known concentrations of human myoglobin standard (10 – 1000 µg/mL) to determine percentage recovery, while the stabilization of myoglobin in a

urine sample during an analytical run was analysed because of its notorious instability in urine (Chen-Levy *et al.*, 2005). Standards ranging from 10 – 1000 µg/mL were added to urine samples before the addition of 1 mL 0.2M NaOH to 5 mL of the spiked sample in a separate sterile tube to replicate standard urine collection protocols. They were diluted 1:10 with 10 mM ammonium acetate pH 7 and analysed every four hours for a 28 hour period at 4°C. This time period was specifically chosen due to the length of each assay (32 minutes) while accounting for as many as 40 samples in any one analytical run.

Stability of myoglobin in a urine sample using the proposed method over an extended period of time was also measured. 10, 100 and 1000 µg/mL human myoglobin standard was added to a urine sample that had been pH corrected (1 mL 0.2M NaOH to 5 mL urine) and then diluted 1:10 with 10 mM ammonium acetate pH 7 before being either: 1) frozen at -80°C, 2) frozen at -20°C or 3) refrigerated at 4°C for one, two, four and eight weeks before thawing and analysis to determine stability and degradation kinetics. All experimental research was conducted in triplicate.

### *Subject*

This method was validated from urine collected from a mixed martial artist following a mixed martial arts (MMA) contest who was informed (Appendix O) of the risks involved in the study before their written consent (Appendix N) was obtained. This was approved by the University of Canterbury Human Ethics Committee, Christchurch, New Zealand.

### *HPLC Analysis*

Refer to Materials and Methods (Chapter 2.2)

### *Statistical Analysis*

Analysis was conducted using the Statistical Package for Social Sciences v. 20. A two-way analysis of variance (ANOVA) using Tukey's test for post-hoc analysis was conducted on all recovery and time course experiments. Statistical significance was set at 0.05.

## **12.3 Results**

Equine skeletal myoglobin provided near identical properties to human skeletal myoglobin as determined by spectrophotometry and HPLC analysis with spectrophotometric detection. We

therefore suggest the use of equine skeletal myoglobin as a standard for clinical measurements by HPLC due to its relative availability and lower purchase price in comparison to human myoglobin.

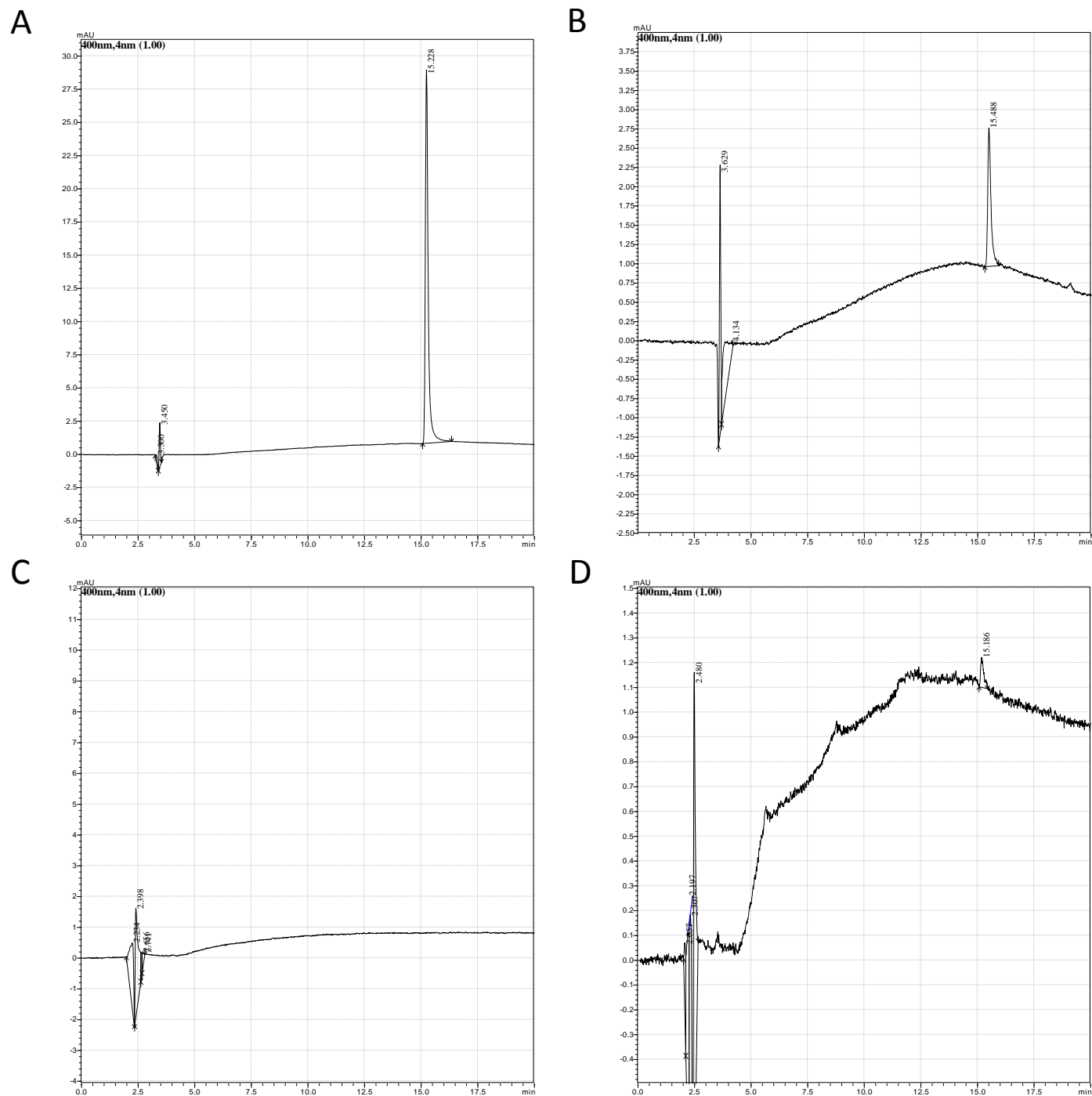
Urinary myoglobin was detected at 400 nm by RP-HPLC eluting at 15.2 minutes with no visible tailing of the peak or interference from neighbouring peaks (Fig. 12.1A and B). After spectrophotometer analysis identified 210, 280 and 400 nm as the optimal absorbance wavelengths (data not shown), spectral analysis of a urine sample spiked with myoglobin by HPLC found 400 nm provided the highest absorbance units, lowest signal to noise ratio and which did not interfere with any other urinary compound (Fig. 12.1B) at that wavelength. Validation of the method identified a single peak following a mixed martial arts contest (Fig. 12.1D) that is clearly undetectable pre-contest (Fig. 12.1C).

The intra- and inter-assay co-efficient of variation for 10, 100 and 1000 µg/mL were 1.49 % and 2.94 %, 0.39 % and 0.70 %, and 0.32 % and 0.79 % respectively, while the assay presented linearity in both human ( $R^2 = 0.998$ ) and equine ( $R^2 = 0.997$ ) over the range of 5 – 1000 µg/mL with a lower detection limit of 2 µg/mL.

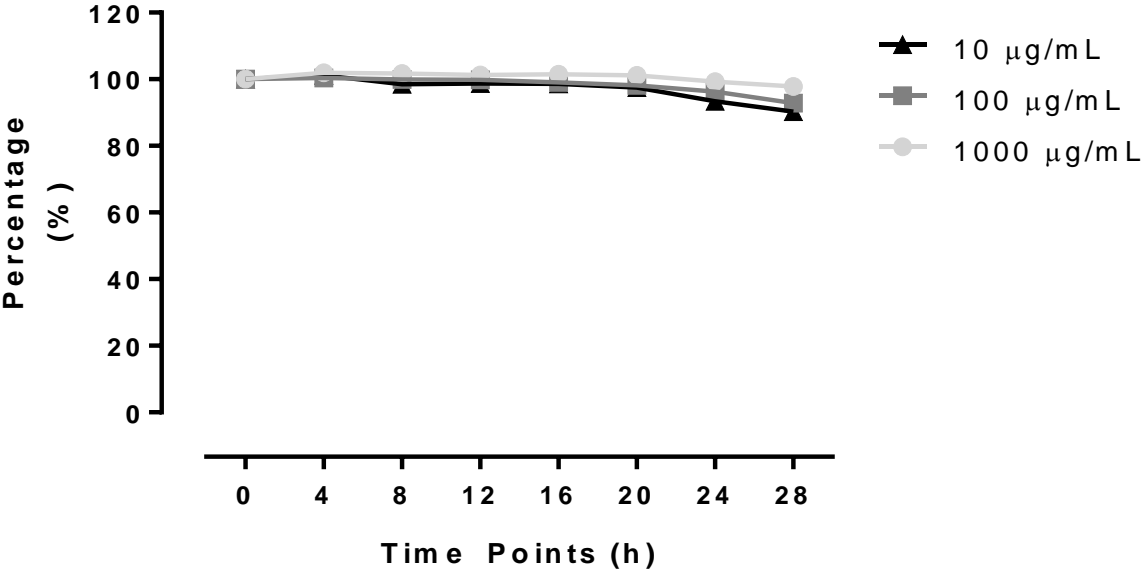
Myoglobin was more stable following alkalization and dilution with 10 mM ammonium acetate pH 7 over a 28 hour analytical run in comparison to purified water (data not shown). It was therefore decided to pursue further stability experiments utilizing this protocol. Myoglobin recovery from spiked urine samples ranged from 96.4 – 102.5 %, while its stability over 28 hours in a urine sample diluted with 10 mM ammonium acetate pH 7 at 4°C decreased by 9.71%, 7.12% and 2.12% for 10, 100 and 1000 µg/mL respectively at the 28 hour time point (Fig. 12.2).

Meanwhile, storage length and conditions has significant effects ( $p < 0.05$ ) on myoglobin stability in urine (Table. 12.1). The longer a sample is stored, the more degradation occurs. However, this loss is attenuated by the storage temperature ( $p < 0.01$ ), while the greater the concentration, the slower the degradation process ( $p < 0.01$ ).





**Figure 12.1.** RP-HPLC analysis of a 100µg/mL myoglobin standard (A), urine sample spiked with 10 µg/mL myoglobin (B), pre-contest MMA urine sample (C), and post-contest MMA urine sample (D) eluting at 15.2 – 15.4 minutes at an absorbance wavelength of 400 nm.



**Figure 12.2.** Myoglobin recovery and stabilization over 28 hours from urine samples diluted 1:10 with 10 mM ammonium acetate pH 7 and spiked with concentrations ranging from 10 – 1000 µg/mL at 4°C.

**Table 12.1.** Storage effect on myoglobin in a pH corrected and diluted urine sample spiked with known concentrations. Data is mean  $\pm$  SD ( $\mu\text{g/mL}$ ).

[Myoglobin] ( $\mu\text{g/mL}$ )	Time Point (weeks)	Conditions		
		4°C	- 20°C	- 80°C
10	1	5.3 $\pm$ 0.2	6.3 $\pm$ 0.3	7.3 $\pm$ 0.01
	2	4.6 $\pm$ 0.3	5.1 $\pm$ 0.2	6.1 $\pm$ 0.5
	4	3.1 $\pm$ 0.1	4.2 $\pm$ 0.01	6.0 $\pm$ 0.2
	8	2.7 $\pm$ 0.1	4.2 $\pm$ 0.02	6.4 $\pm$ 0.01
100	1	81.0 $\pm$ 0.3	81.2 $\pm$ 0.04	86.0 $\pm$ 0.1
	2	83.4 $\pm$ 0.1	83.2 $\pm$ 0.7	91.4 $\pm$ 2.0
	4	77.3 $\pm$ 0.8	71.6 $\pm$ 3.9	82.3 $\pm$ 0.1
	8	72.3 $\pm$ 0.1	67.0 $\pm$ 1.4	77.0 $\pm$ 0.5
1000	1	975.3 $\pm$ 1.2	998.6 $\pm$ 22.0	987.5 $\pm$ 36.3
	2	963.4 $\pm$ 20.6	980.9 $\pm$ 7.6	1020.4 $\pm$ 1.1
	4	947.9 $\pm$ 4.6	952.7 $\pm$ 6.8	980.0 $\pm$ 2.2
	8	924.5 $\pm$ 18.0	941.9 $\pm$ 7.5	977.0 $\pm$ 17.1

## 12.4 Discussion

Myoglobin is typically quantified in urine through sensitive and rapid immunoassays (Wu *et al.*, 1994) as well as several forms of HPLC (Powell *et al.*, 1984). With intra- and inter-assay variations in accordance with the accepted range for a reliable and repeatable assay, we have demonstrated a suitable, reliable and rapid (32 minutes) alternative RP-HPLC method for the quantification of urinary myoglobin following serious trauma. Detection of myoglobin at an absorbance of 400 nm produced a clear visible peak with no observed tailing or interference from neighbouring urinary analytes allowing for clear interpretation, while the addition of 1 mL 0.2 M NaOH to 5 mL urine before dilution in 10 mM ammonium acetate pH 7 seemed to alleviate any stabilization concerns that have been previously noted over a 24 hour period (Chen-Levy *et al.*, 2005). The mechanism of how this alkalization improves myoglobin

stability has not yet been fully elucidated. It is believed however, to revolve around myoglobin's isoelectric point where increasing the pH beyond 6.9 reduces its precipitation (King *et al.*, 2010) and further prevents the dissociation of myoglobin into its smaller nephrotoxic components (Alterman *et al.*, 2007). This issue becomes particularly imperative when assessing medical issues pertaining to muscle damage quantification and the potential for false-negative results arising from myoglobin degradation in the bladder (de Waard and van't Sant 2009).

In spite of relative stability over 28 hours using this protocol which alleviates any potential issue arising from the extended HPLC assay time, the time course experiments suggest urinary myoglobin be analysed immediately following collection and alkalization to avoid any potential degradation loss when concentrations are not known. These results expand upon the previous findings that found alkalized urine stored at 4°C decreases up to 50 % within a week (de Waard and van't Sant 2009). Whilst some compounds remain stable for months at - 80°C (Garde and Hansen 2005), urine samples containing < 1000 µg/mL myoglobin clearly do not for long periods of time and should therefore avoid freeze-thawing for optimal analysis. Samples should be collected, alkalized and analysed, or if need be, diluted accordingly with a pH 7 buffer and stored promptly at - 80°C upon presentation. The dilution factor can of course be altered depending upon myoglobin concentration or concern for column life expectancy.

Myoglobin's detection and recovery from urine samples using this method is comparable to similar RP-HPLC methods (Oellingrath *et al.*, 1990) that provide a simple and cost-effective assay using UV detection for values in a clinically relevant range. Its use in a clinical and exercise context for diagnosis and observational purposes should therefore consider sample pH stabilization and dilution in 10 mM ammonium acetate pH 7 followed by analysis within 20 hours at 4°C to utilize this method to its fullest capacity. This is highly feasible considering the non-invasive, non-burdening nature of this method with no need for venepuncture, the faster elimination kinetics of myoglobin compared to the sometimes delayed response of common muscle damage makers such as creatine kinase (Lappalainen *et al.*, 2002), and the ease of installation of the measurement apparatus within a laboratory or hospital setting. Moreover, with commercial ELISA assays having upper detection limits of ~ 1 µg/mL, the linearity of this method from 5 – 1000 µg/mL and lower detection limit of 2

µg/mL provides a significant alternative for the quantification of a muscle damage marker. These properties are similar to that reported by anion exchange (Powell *et al.*, 1984) and two-dimensional liquid chromatography-electrospray ionization mass spectrometry (Mayr *et al.*, 2006) which negates the dilution requirement for absolute concentration determination, and which also avoids unnecessary repeated analysis.

Although it does not have the sensitivity limit of 10 ng/mL that prior chemiluminescence detection has shown (Maltsev *et al.*, 1987), the concentrations in the linear range are more clinically relevant and similar to that previously noted (Powell *et al.*, 1984). Feinfeld *et al.* (Feinfeld *et al.*, 1992) have observed patients with acute rhabdomyolysis exceeding concentrations of 1 µg/mL, while previous studies have suggested patients with values exceeding 20 µg/mL are more at an increased risk of decreased renal function (Loun *et al.*, 1996; Wu *et al.*, 1994). Additionally, values as high as 32.9, 410 and 3000 µg/mL have been observed following crush trauma (Genthon and Wilcox 2014), strenuous military training (Smith 1968), and following myocardial infarction (Powell *et al.*, 1984), respectively, while values reaching 52 µg/mL have been observed in competitors following a marathon (Schiff *et al.*, 1978). Therefore, in an exercise and/or serious trauma scenario where muscle damage is expected to rise significantly, this assay may provide the most cost-effective and operator-friendly option for simple and rapid quantitation for both small and large cohorts suffering severe skeletal muscle stress in a range that is widely accepted as “clinically relevant”.

### *Conclusion*

The quantification of urinary myoglobin using the described RP-HPLC method provides a precise, simple and cost-effective method for muscle damage quantitation in a clinically relevant range. The detection of myoglobin using this method in a mixed martial arts competitor identifies severe structural damage following a contest validating its value, while immediate alkalization and dilution in a pH 7 buffer alleviates any stabilization concerns over 24 hours. Through its simplicity, non-invasiveness and speed of analysis, this method can further be used for informing the recovery of patients suffering from such varied conditions as crush injury, myocardial infarction and rhabdomyolysis.

# Chapter 13

## Effect of Cryotherapy on the Physiological Stress Response and Blood Derived Mononuclear Cell Activation Following a Mixed Martial Arts Contest: A Pilot Study

### Abstract

The purpose of this study was to investigate the structural stress following a mixed martial arts contest and whether cryotherapy attenuates inflammatory and muscle damage markers and *in vitro* monocyte/T-cell activation. Urine was collected from 10 elite mixed martial artists before, immediately post and at 1, 2, 24 and 48 hours following a contest. Myoglobin was analysed using RP-HPLC, while urinary neopterin and total neopterin (neopterin + 7,8-dihydroneopterin) were measured by SCX-HPLC. Cold water immersion and passive recovery were compared using changes in these markers, while a cooling treatment tested total neopterin production in  $\gamma$ -interferon and PMA stimulated monocytes/T-cells. Myoglobin and neopterin significant increased ( $p < 0.05$ ) at one and 24 hours respectively, total neopterin significantly increased ( $p < 0.05$ ) at one hour post for the passive group only, and significant individual variation was observed in all markers ( $p < 0.01$ ). Cold water immersion attenuated total neopterin production ( $p < 0.05$ ) while cooling significantly reduced total neopterin production in PMA stimulated T-cells ( $p < 0.01$ ). Mixed martial arts causes significant muscle damage and inflammation akin to similar other high intensity contact sports. Similarly, cold water immersion may be a useful recovery intervention through peripheral blood flow reduction, vasoconstriction and direct immune cell suppression when significant structural damage occurs following exercise.

### 13.1 Introduction

Mixed martial arts (MMA) is a relatively new sport that incorporates several forms of martial arts from Jiu-Jitsu to kick-boxing. It has gained notoriety through its combative nature which has drawn interest from multiple disciplines and champions of their own respective martial art. In a sport that revolves around grappling, striking and wrestling with impacts known to generate 90 – 120 G (Schwartz *et al.*, 1986), the level of associated muscle damage and

inflammation is relatively unexplored, despite studies having identified MMA fights having injury rates ranging from 23.6 - 40.3 % to at least one of the competitors (Bledsoe *et al.*, 2006; Ngai *et al.*, 2008).

Whilst several studies have been conducted on the diverse martial arts disciplines (Capranica *et al.*, 2012), little has been conducted specifically on MMA. Other physical impact sports such as rugby union, rugby league, American football and jiu-jitsu have been shown to cause substantial increases in markers of physiological stress (Hoffman *et al.*, 2002; Lindsay *et al.*, 2014b; McLellan *et al.*, 2010; Santos *et al.*, 2012), which suggests the force and frequency of the impacts accompanying this sport will induce similar trauma. Buse (2006) identified 14.1 % of all MMA contests end because of musculoskeletal stress while jiu-jitsu, Tae Kwon Do and Greco-Roman wrestling training/competition cause significant elevations in haematological stress parameters (Brandao *et al.*, 2014; Maffulli *et al.*, 1996; Santos *et al.*, 2012). It is therefore plausible that significant elevations in oxidative stress, inflammation and muscle damage may transpire.

Neopterin (NP), total NP (NP + 7,8-dihydroneopterin) and myoglobin are key physiological markers of these respective processes which have been used to assess exercise induced physiological stress (Lindsay *et al.*, 2015a; Lindsay *et al.*, 2014b) and whose measureable changes present credible and reliable information pertaining to immune system activation and loss of muscle membrane integrity. Innate inflammation is characterised by T-cell stimulation of macrophages through the release of  $\gamma$ -interferon which causes monocytes and macrophages to generate 7,8-dihydroneopterin; a potent antioxidant in its own right (Giese *et al.*, 2000) which may protect the macrophages from their own oxidants during inflammation.

Recovery from impact induced physiological trauma is critical for sustained training and improved performance without risking the development of non-functional overreaching or over-training syndrome. Cold water immersion (CWI) has been shown to be an effective post-exercise intervention following impact trauma (Gill *et al.*, 2006) and resistance training induced muscle damage (Roberts *et al.*, 2014) that is more readily available and similarly efficient at cooling (Costello *et al.*, 2014a) in comparison to more modern whole body cryotherapy (WBC) strategies. Commonly used for its analgesic properties and shown to reduce muscle temperature by  $\sim 6 - 9$  °C (Ihsan *et al.*, 2014; Roberts *et al.*, 2014), CWI is

capable of alleviating symptoms of delayed onset muscle soreness (DOMS), improve muscle power recovery, reduce post-exercise increases in creatine kinase (Leeder *et al.*, 2011) and enhance the adaptation response through mitochondrial biogenesis (Ihsan *et al.*, 2014). These effects are thought to occur through reduced peripheral blood flow, vasoconstriction (Park *et al.*, 1999) and hydrostatic pressure (Choo 2014). However, the direct effect of cryotherapy on immune cell activation *in vitro* is to the best of our knowledge currently unknown (Chow *et al.*, 2015) and a potential alternative explanation for these observed effects. Icing has been shown to retard macrophage abundance by ~ 1-2 days (Takagi *et al.*, 2011) following muscle damage, however the potential to modulate the cellular inflammatory response by cold exposure remains un-tested. Furthermore, there is equivocal evidence surrounding the positive effect of CWI on systemic markers of inflammation and muscle damage (Fragala *et al.*, 2015; Takeda *et al.*, 2014) which suggests further investigation is required.

Increasing popularity and participation in MMA warrants further research to understand the stresses these athletes endure and how different recovery protocols may be used to improve recovery and performance. The purpose of this research is to quantify the level of inflammation (total NP), oxidative stress (NP) and muscle damage (myoglobin) following a simulated mixed martial arts contest and identify whether CWI is more effective at attenuating these physiological responses in comparison to passive recovery. It also hopes to provide an *in vitro* understanding on the effect of cryotherapy on monocyte/T-cell activation and determine whether this can offer an alternative explanation to vasoconstriction in the hypothesized reduction in total NP following CWI.

## 13.2 Methods

### *Subjects*

Ten elite amateur and professional mixed martial arts athletes volunteered for the study (Age:  $27.3 \pm 3.3$  y; Mass:  $79.5 \pm 0.5$  kg; Height:  $1.77 \pm 0.04$  m) with a combined MMA record of  $3.9 \pm 3.4$  wins and  $2.1 \pm 1.7$  losses. The experimental protocol was approved by the University of Canterbury Human Ethics Committee (Christchurch, New Zealand) and all subjects were informed (Appendix O) of the risks involved in the study before their written consent was obtained (Appendix N).



*Experimental Protocol*

A mixed martial arts contest can last anywhere from a few seconds to 25 minutes. As a result of this uncontrollable circumstance, each subject was weight matched with an opponent in a full contact contest lasting three rounds of five minutes. To gain a complete understanding of potential structural stress accompanying a longer length contest regardless of how many times a participant was “beaten”, the entire contest duration was completed. If a participant received a lesion or any form of concussion that was deemed too dangerous to continue with (diagnosed by a clinical physician onsite), the contest was halted immediately.

All subjects undertook a 24 hour rest period before and 48 hours after the contest day, and abstained from any form of exercise other than the contests themselves. All subjects consumed approximately 1L H<sub>2</sub>O, a carbohydrate-protein beverage, and slept for at least eight hours following the contest. They each completed their usual post-contest recovery protocol which included either CWI immediately post and on the next two consecutive mornings (~ 12 and 36 hours post-contest) (n = 5) or passive recovery (n = 5). The CWI protocol used by the subjects involved submerging themselves, except the neck and head, for 15 minutes in a 10°C bath which is commonly used in CWI studies (Roberts *et al.*, 2014) and verified to reduce muscle temperature, blood flow, energy demand, micro-vascular perfusion and metabolic activity following exercise (Broatch *et al.*, 2014; Ihsan *et al.*, 2014; Ihsan *et al.*, 2013; Mawhinney *et al.*, 2013).

Urine samples were collected pre, immediately post, and one, two, 24 and 48 hours post-contest. Each participant provided a specimen mid-stream (from the bladder) into a 70 mL collection pottle. All samples were placed on ice immediately and transported to the laboratory for analysis. For myoglobin quantification, 5 mL of urine was aliquoted at time of collection into a sterile 15 mL tube containing 1 mL 0.2 mol/L NaOH that adjusted the pH to approximately 7 – 9 as described previously (Lindsay *et al.*, 2014b).

*Urine Sample Preparation, HPLC Analysis, Specific Gravity and Video Analysis*

Refer to Material and Methods (Chapter 2.2)

### *Cell Culture and Sample Preparation*

Mononuclear cells (monocytes and lymphocytes) were prepared from whole human blood supplied by the New Zealand blood service under Human Ethics approval by centrifugation with lymphoprep as previous described (Giesege *et al.*, 2010). Cells were plated in RPMI1640 supplemented with 10 % human serum, at  $10 \times 10^6$ /well and incubated at 37°C in 5% CO<sub>2</sub>. After 24 hours, cells were either treated with 5 µM phorbol myristate acetate (PMA) (dissolved in DMSO) or 500 units γ-interferon. PMA stimulates T-cells generate γ-interferon while the addition of γ-interferon stimulates the monocytes directly. After one hour incubation at 37°C, selected plates were placed in a fridge at 4°C for one hour before returning to the 37°C incubator. At indicated time points during the treatments 100 µL of media was removed for total neopterin analysis.

Neopterin analysis for cell media samples, an equal volume of acetonitrile (100 µL) and 40 µL acidic iodide solution (5.4% I<sub>2</sub>/10.8% KI in 1 M HCl) were added to each sample (Flavall *et al.*, 2008). Each was vortexed and incubated in the dark for 15 minutes. 20 µL of 0.6 M ascorbate was then added and followed by centrifugation at 18,000 g. For analysis of cell media where the proteins were precipitated with acetonitrile, the mobile phase also contained 10% acetonitrile.

### *Statistical Analysis*

The effect of contest variables (number and type of impacts, contest outcome and recovery protocol) on the change in biomarker concentrations were tested in a linear mixed effects model fitted with restricted maximum likelihood, conducted in the lme4 package (Bates *et al.*, 2014) in R version 3.1.1 (Team 2014). P values for coefficients of fixed effects were calculated using Satterthwaite's method of denominator synthesis, conducted in the lmerTest package (Kuznetsova *et al.*, 2013) for R. Each marker was analysed as the response variable in a separate model. The fixed predictors in each model were number and type of impacts sustained by the participant during the contest, outcome of the contest (win or loss) and recovery protocol. Participant identity was included as crossed random effects to account for the non-independence of marker measures from each participant. A two-way analysis of variance (ANOVA) was similarly used to compare differences in total NP production from a

mixed cell culture incubated at different temperatures with Tukey's test post-hoc analysis. Data is presented at mean  $\pm$  SEM unless otherwise stated.

### 13.3 Results

Notational analysis identified an average ( $\pm$  SD) of 53.5 (30.5) punches, 15 (9.7) kicks, and 3.3 (2.95) takedowns sustained per contest.

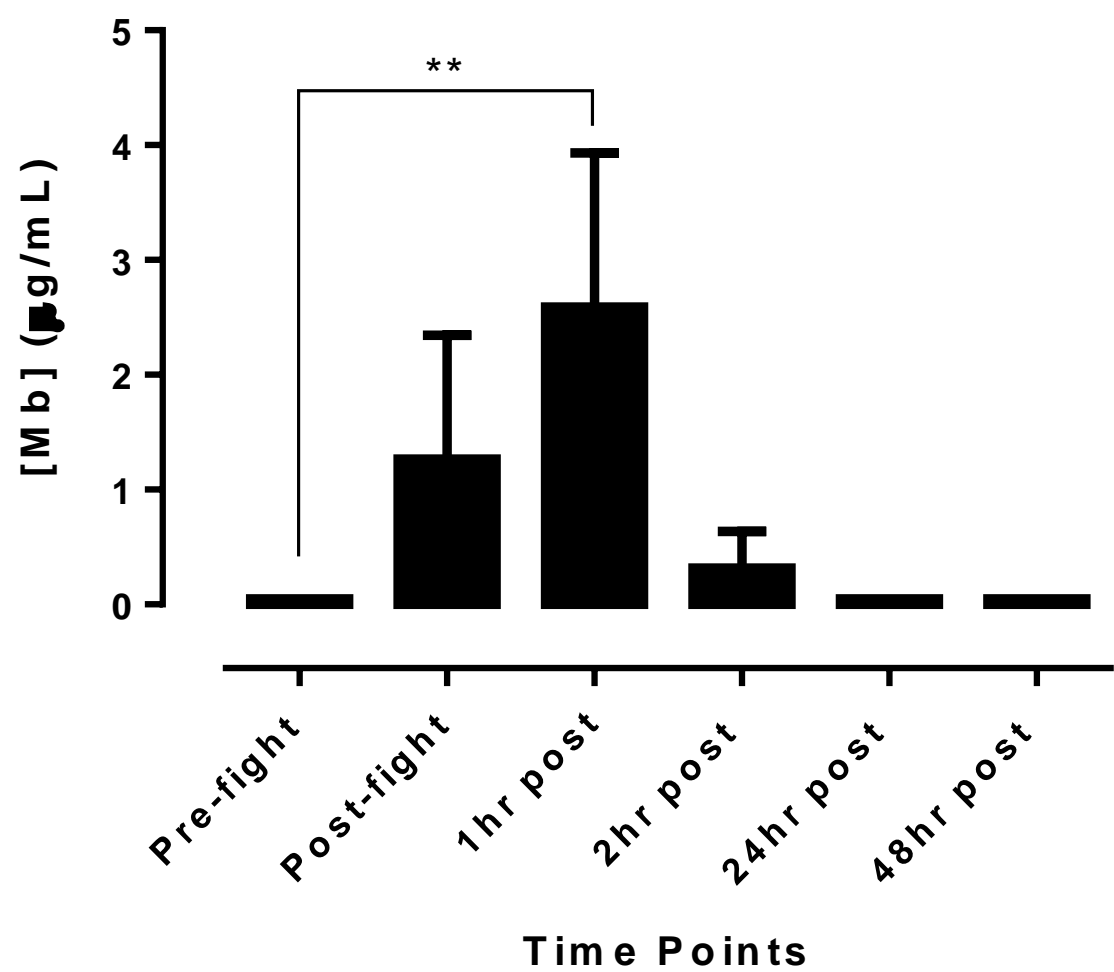
Urinary myoglobin increased significantly ( $p = 0.005$ ,  $n^2_{\text{partial}} = 0.39$ ) above pre-contest concentrations at one hour post (Fig. 13.1) and returned to undetectable levels by 24 hours. It was detected in five of the ten participants with concentrations ranging from 0.95 – 13.24  $\mu\text{g/mL}$ . Concentrations were not correlated to the number or type of impacts a participant experienced or the outcome of the contest.

Urinary NP significantly increased ( $p = 0.02$ ,  $n^2_{\text{partial}} = 0.35$ ) after one and 24 hours post-contest ( $p = 0.04$ ,  $n^2_{\text{partial}} = 0.48$ ). Concentrations remained marginally elevated ( $p = 0.0894$ ,  $n^2_{\text{partial}} = 0.33$ ) above pre-contest levels after 48 hours (Fig. 13.2A) with significant ( $p < 0.01$ ) inter-individual variation (Fig. 13.2B). There was also a moderate correlation ( $r = 0.51$ ) between the percentage change in NP within the first two hours post-fight and the concentration of myoglobin.

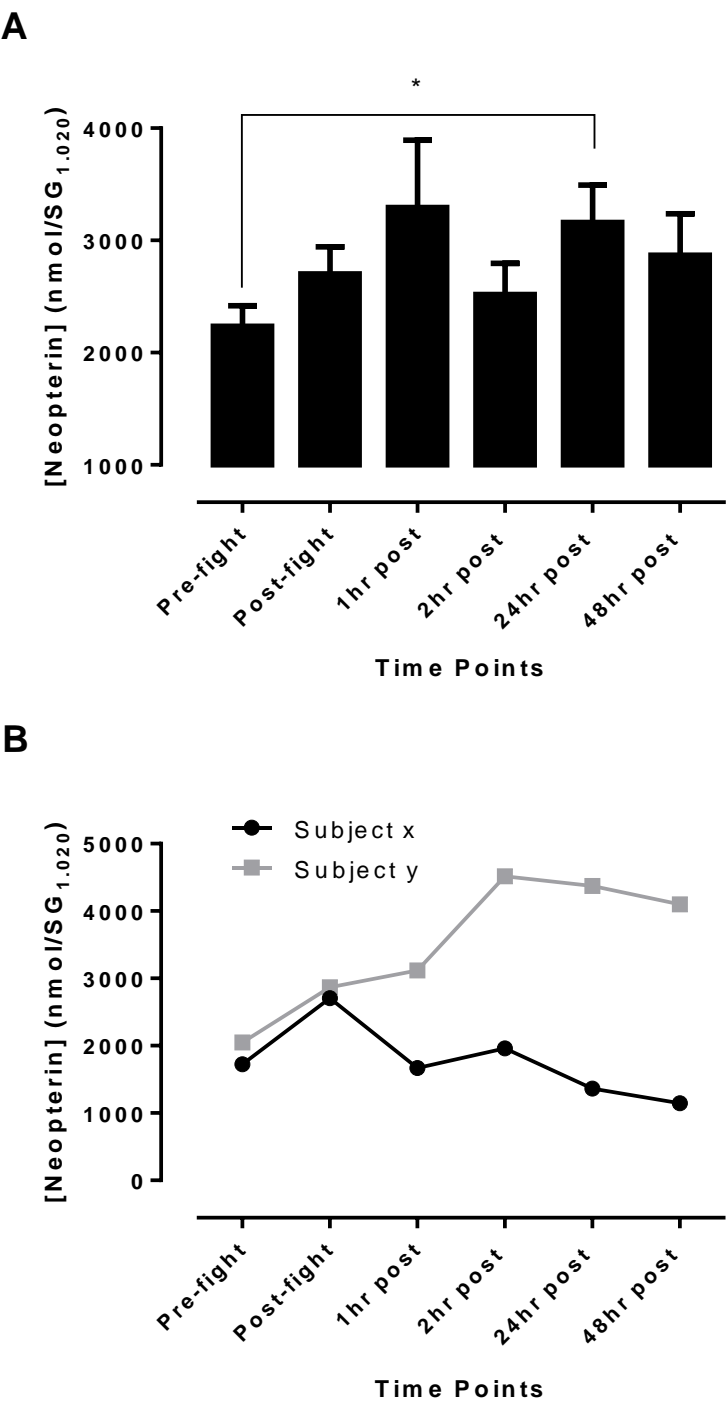
Total NP analysis provided insufficient evidence that the MMA contest caused any meaningful change (Fig. 13.3A), however there was a significant increase in the passive recovery group ( $p < 0.01$ ,  $n^2_{\text{partial}} = 0.50$ ) and a significant ( $p = 0.01$ ) inter-individual variation (Fig. 13.3B). Number and type of impacts and outcome of the contest had no effect on NP and total NP concentrations, while no one fight seemed to cause more or less change ( $p > 0.05$ ).

Recovery intervention analysis indicates urinary NP concentrations was marginally increased ( $p = 0.07$ ,  $n^2_{\text{partial}} = 0.55$ ) in the CWI group at one hour post (Fig. 13.4A) in comparison to the passive recovery group. However, urinary total NP was significantly lower ( $p = 0.04$ ,  $n^2_{\text{partial}} = 0.63$ ) at one hour post-contest for the participants completing a CWI immediately following the contest in comparison to passive recovery (Fig. 13.4B). At 24 and 48 hours post-contest, no difference was observed between the groups.

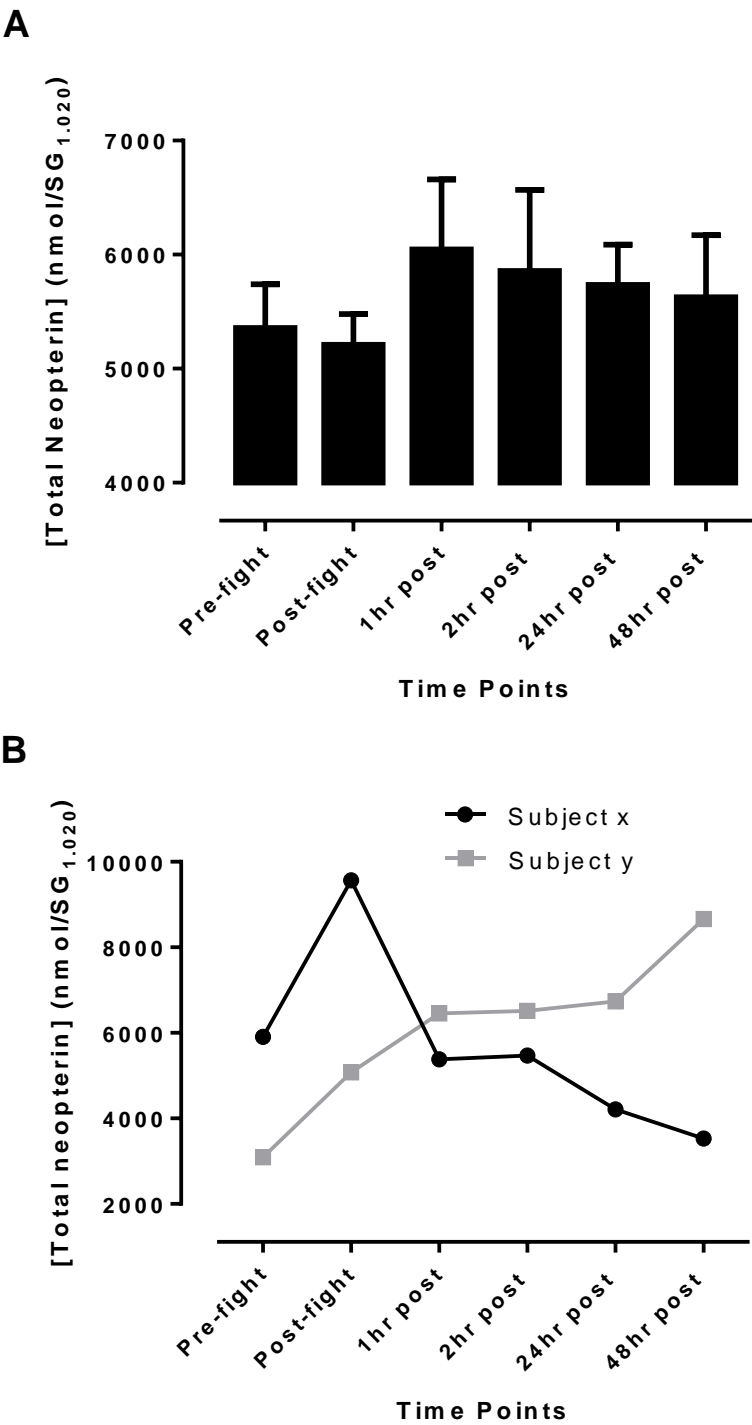
The incubation of the blood derived mononuclear cells at 4°C did not visibly alter them morphologically (data not shown). There was no evidence to suggest ( $p = 0.19$ ,  $n^2_{\text{partial}} = 0.01$ ) incubating at 4°C had any effect on total NP production following  $\gamma$ -interferon incubation over 24 hours compared to 37°C (Fig. 13.5A). There is however, sufficient evidence ( $p < 0.01$ ,  $n^2_{\text{partial}} = 0.70$ ) that total NP production is reduced following incubation with PMA at 4°C compared to 37°C (Fig. 13.5A). When this change was observed as a time course, (Fig. 13.5B) there was an immediate reduction in total NP production one ( $p < 0.05$ ,  $n^2_{\text{partial}} = 0.94$ ), 12 ( $p < 0.05$ ,  $n^2_{\text{partial}} = 0.95$ ), and 24 hours ( $p < 0.05$ ,  $n^2_{\text{partial}} = 0.86$ ) after cryotherapy. The evidence does suggest there was a minor difference between the treatments at 3 ( $p = 0.06$ ,  $n^2_{\text{partial}} = 0.80$ ) and 6 ( $p = 0.06$ ,  $n^2_{\text{partial}} = 0.71$ ) hours post as well, while no difference after 48 and 96 hours.



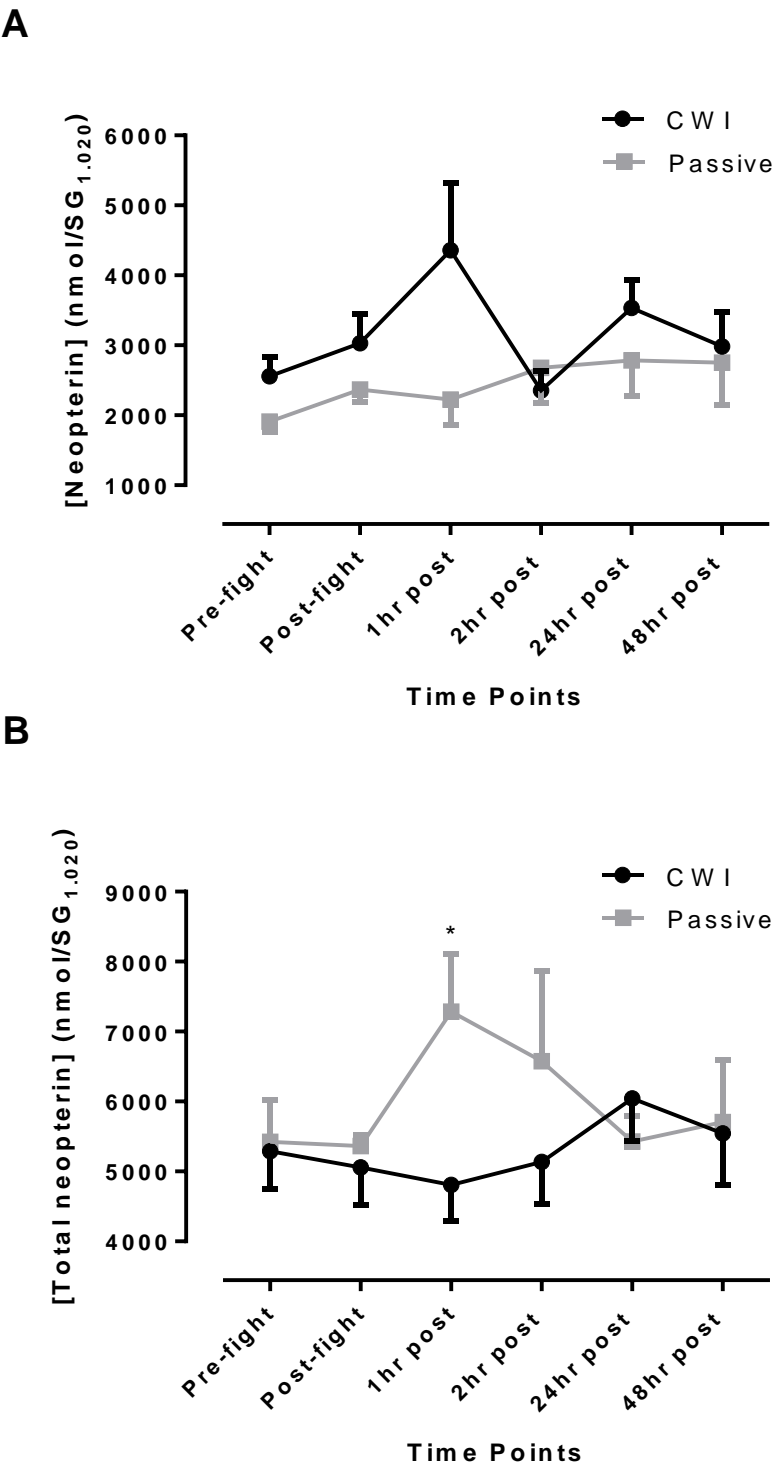
**Figure 13.1.** Urinary myoglobin time course changes. Data is presented as mean ± SEM. \*\*  
p < 0.05



**Figure 13.2.** Urinary neopterin time course changes for the group (A) and selected individuals with a varied response (B) following the contests. Data is presented as mean ± SEM. \*  $p < 0.05$

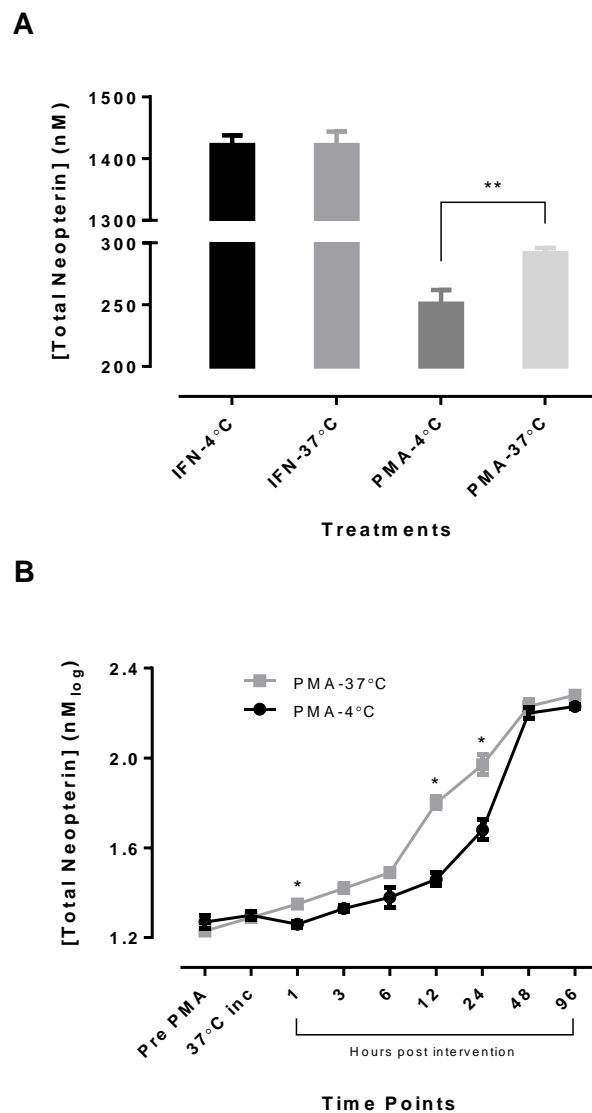


**Figure 13.3.** Urinary total neopterin time course changes for the group (A) and selected individuals with a varied response (B) following the contests. Data is presented as mean ± SEM.



**Figure 13.4.** The effect of CWI and passive recovery on changes in urinary neopterin (A) and total neopterin (B). Data is presented as mean  $\pm$  SEM. \*  $p < 0.05$ .





**Figure 13.5.** Effect of cold treatment on total neopterin production by mononuclear cells. Blood derived mononuclear cells consisting of a mixture of T-cell and monocytes were stimulated with either 500 units  $\gamma$ -interferon or 5  $\mu$ M PMA. A) After one hour of incubation selected plates (containing  $10 \times 10^6$ /well) as indicated were either transferred to a 4°C fridge for 1 hour before being returning to the 37°C incubator. The remaining plates were left at 37°C for the entire 24 hours before media was sampled for total neopterin release. B) Release of total neopterin into the media was measured at indicated time points following stimulation with 5  $\mu$ M PMA using the cold treatment specified for A. Each data point is the mean of three individual wells for each time point consisting of  $5 \times 10^6$  cells/well. Data is log transformed and presented as mean  $\pm$  SEM. \*  $p < 0.05$ .

### 13.4 Discussion

The cold water immersion protocol utilized in this study attenuates the initial elevations in structural stress biomarkers accompanying a mixed martial arts contest. This is in contrast to the lack of difference in certain haematological parameters measured following CWI and other high-intensity exercise (Broatch *et al.*, 2014; Jajtner *et al.*, 2014) suggesting further research into a standardized approach is still required. With myoglobin concentrations known to remain elevated up to 24 hours post-exercise (Neubauer *et al.*, 2008), its return to non-detectable levels at 24 hours suggests both protocols had a positive effect on intramuscular properties comparable to that previously noted in jiu-jitsu (Júnior *et al.*, 2014; Santos *et al.*, 2012) and other high intensity exercise (Minett *et al.*, 2014; Roberts *et al.*, 2014). Similarly, the significant elevation in total NP following the contest seems to be attenuated at least in the hours immediately following a contest, while the consumption of a protein and carbohydrate drink, water and at least eight hours sleep does not affect the immediate inflammatory response, but does seem to be substantial enough to induce recovery 24 - 48 hours post-exercise as previously noted (Groen *et al.*, 2012; Millard-Stafford *et al.*, 2005; Valentine *et al.*, 2008).

The significant reduction in urinary total NP and likelihood of reduced skin and core temperature in the CWI group following this particular protocol (Costello *et al.*, 2012) that is suggested for positive analgesic and vaso-constrictive effects (Bleakley and Hopkins 2010), proposes a reduction in immune cell transportation to sites of tissue damage. This theory is solidified through an observed restriction in red blood cell concentration and microcirculation following CWI (Costello *et al.*, 2014b). Furthermore, WBC may reduce leukocyte circulation through a drop in core temperature and reduced expression of soluble intercellular adhesion molecule-1 (sICAM-1) (Ferreira-Junior *et al.*, 2014) although this theory has been recently argued (Dugue 2015).

Our data offers an alternative mechanism that may parallel these previously identified properties of CWI. The cooling of a mixed cell culture of monocytes and T-cells (blood derived mononuclear cells) incubated with PMA and  $\gamma$ -interferon suggests it does not have any meaningful effect on  $\gamma$ -interferon stimulated monocyte activation, but rather suppresses  $\gamma$ -interferon production by T-cells. This is clearly indicative of a latent effect that suggests cryotherapy may concurrently suppress the activation of the resident immune cells both

immediately (corroborates the *in vivo* suppression following the MMA contest) and for a period of up to 24 hours. So while cryotherapy acts to attenuate immune cell transportation, it may simultaneously diminish their ability to generate a potential excessive inflammatory response. Whilst this provides the first *in vitro* evidence pertaining to the cooling effect on immune cells responsible for tissue regeneration, future research into cryotherapy techniques needs to consider the impact this may have on the cryotherapy phenomena. It has to be noted however, that macrophage activation is imperative for the muscle regenerative process (satellite cell activation, proliferation and differentiation) (Kharraz *et al.*, 2013) which opens up debate to the validity of cryotherapy treatments in the role of adaptation and their effectiveness following exercise induced muscle damage (Yamane *et al.*, 2015). This CWI protocol however, did not seem to retard the adaptation and regenerative process at 24 and 48 hours post-exercise in the CWI group in comparison to passive recovery.

Cold water immersion may also cause an immediate change in oxidative status which is in contrast to the positive effects on the oxidant/antioxidant balance of WBC following exercise (Sutkowy *et al.*, 2014). This contrast may be explained by an increase in reactive oxygen species through elevated mitochondrial biogenesis (increased expression of mRNA peroxisome proliferator-activated receptor gamma coactivator-1 $\alpha$  (PGC-1 $\alpha$ )) following CWI (Ihsan *et al.*, 2014), and the adaptive response of mitochondrial thermogenesis to cold exposure (Rakobowchuk *et al.*, 2008). Its use ~ 12 and 36 hours following exercise however, does not seem to have any further effect on an inflammatory response which clarifies how imperative immediate post-exercise recovery intervention is for reducing swelling and inflammation, if that be the desired effect.

This study is also the first to report changes in urinary NP, total NP and myoglobin following a MMA contest. The contests caused significant muscle damage, inflammation and oxidative stress in both group and individual contexts which is similar to that previously noted in other physical impact sports (Lindsay *et al.*, 2015c) and traditional martial arts (Brandao *et al.*, 2014). More importantly, pronounced individual variation (randomly selected from subjects with opposing changes) was identified following the MMA contest which we have previously reported in amateur and professional rugby players (Lindsay *et al.*, 2014b; Lindsay *et al.*, 2015c) where a similar pattern of disparity diagnoses the need to treat every individual athlete as their own entity, and solidifies the use of biochemical analysis for quantification of

exercise induced stress. This is particularly important for management and providing the best opportunity for each fighter to respond and prepare optimally for a competition rather than relying solely on group data that often masks the individual physiological response.

Urinary myoglobin detection in five of the ten participants provides further conclusive evidence that MMA causes significant structural damage in a clinically relevant range (Feinfeld *et al.*, 1992), albeit at a considerably lower level than other impact sports (Lindsay *et al.*, 2015c). Values exceeding 1 and 20  $\mu\text{g/mL}$  are related to rhabdomyolysis susceptibility (Feinfeld *et al.*, 1992) and renal dysfunction (Wu *et al.*, 1994), highlighting the severity of intramuscular stress associated with MMA and the necessity of physiological stress quantification for health and recovery maintenance of elite athletes. Furthermore, the failure to identify myoglobin in all participants suggests they are well adapted to the stress of MMA or had concentrations in an undetectable range. The former is highly conceivable due to the six days/week training/competing schedule they complete and perhaps explains why myoglobin may become drastically increased following unaccustomed trauma under different circumstances (Smith 1968).

Meanwhile, the elevation above pre-contest concentrations in urinary total NP for certain participants is indicative of immune system and macrophage activation. Its sustained elevation above pre-contest levels at 24 and 48 hours for a select few participants represents either a sustained inflammatory effect or a delay in macrophage activation as a result of the CWI similar to that previously identified in rat muscle (Takagi *et al.*, 2011). Whilst impact numbers are significantly greater in MMA than other impact sports (Lindsay *et al.*, 2015c), the duration and type of exercise may play a more significant role in macrophage activation, hence the lower values observed in this study cohort. Moreover, impacts do not seem to correlate with the extent of muscle damage and inflammation which is in contrast to other impact related sports (Smart *et al.*, 2008). Contest duration and adaptation may play a major role in this discrepancy.

The MMA contest also caused a significant change in oxidative status. This may be directly attributed to the impacts and associated inflammatory response, or simply the metabolism increase known to generate 10.2 – 20.7  $\text{mmol/L}^{-1}$  lactate following a MMA bout (Amtmann *et al.*, 2008). The sustained elevation of neopterin represents a 24 – 48 hour recovery period and potential for impaired recovery following a MMA contest due to its ability to identify

athletes in a continual state of recovery or over-training (Jakeman *et al.*, 1995). Meanwhile, the correlation with myoglobin offers an alternative hypothesis to hypochlorite (Widner *et al.*, 2000) oxidation of 7,8-dihydroneopterin to neopterin (NP) as previously reported (Lindsay *et al.*, 2015b), while also solidifying myoglobin's oxidative potential (Moore *et al.*, 1998) and the suggested feedback loop intertwining muscle damage and oxidative status (Lindsay *et al.*, 2015b). This relationship suggests the greater the degree of muscle damage, the greater the likelihood of elevated oxidative stress.

### *Conclusion*

A mixed martial arts contest causes severe structural damage (myoglobin), inflammation (total neopterin) and change in oxidative status (neopterin) with the latter partially dependent on degree of muscle damage. CWI attenuates the immediate post-exercise inflammatory response, however its use long term may inhibit adaptation and the regenerative process (Roberts 2014). Meanwhile, biochemical analysis of these athletes through non-invasive protocols may provide a comprehensive alternative to subjective and qualitative measures such as questionnaires about perceived soreness and fatigue and separate individualistic physiological responses.

### *Key Findings*

- An MMA contest causes significant elevations in inflammation, oxidative stress and muscle damage
- Cryotherapy attenuates the immediate post-exercise inflammatory response in MMA and suppresses *in vitro* T-cell activation
- Cryotherapy did not retard the adaptation response following a MMA contest
- Degree of muscle damage may propagate the level of oxidative stress

# Chapter 14

## General Discussion

### 14.1 GENERAL DISCUSSION

The purpose of this thesis was to identify unique, non-overlapping, and meaningful psychophysiological biomarkers that were capable of providing a quantitative stress assessment of a professional athlete to monitor and manage their health and recovery in order to optimise performance. It has provided a unique insight into the stresses of several physically demanding sports which demonstrates this type of assessment is warranted in the management of athletes in professional settings. The five studies conducted each provide a distinctive addition to the current literature on sports exercise physiology and biochemistry. The development of professional relationships was pertinent to the success of this thesis. With key people from the New Zealand Rugby Union, intensive care unit (ICU) and array of physiologists and biochemists involved, the growth and potential was realised.

The principal outcomes from this research were:

- Development and validation of a set of non-overlapping, specific and sensitive biomarkers capable of comprehensively identifying and assessing exercise-induced psychophysiological stress.
- Psychophysiological stress assessment of elite and professional athletes in a non-invasive, non-disruptive and stress free manner.
- Identification of intra- and inter-individual variation due undoubtedly to the individual genetic and physiological characteristics of each subject.

A reliable assay for the quantification of selected urinary pterins was initially required that could be used on a repeatable basis for the detection of inflammation and oxidative stress. Following its development, no significant physiological changes were observed during a week of resistance training in competitive natural body builders; however significant individual variation existed. Furthermore, specific gravity was determined to provide comparable results to creatinine for urine volume correction which was further corroborated in the second study on elite amateur rugby. This study identified each biomarker peaking

immediately after the game and returning to pre-game concentrations 17 hours post-game. This set the foundation for reliable future sample collection whilst simultaneously solidifying each biomarker as specific and sensitive to the rigors of rugby union. In conjunction with this pilot study, measurement of respiratory changes and lung inflammation were considered as another possible gauge of exercise-induced stress. Taken from a clinical setting in the ICU, the use of a ventilator provided a new means of use for this type of equipment, alongside identifying post-exercise lung inflammation in selected individuals. Whilst the time course for this experiment was not ideal, there is potential for future exercise related applications.

With conclusive evidence regarding each biomarkers response to rugby union, the remaining studies identified a range of acute and chronic physiological and psychological changes in professional rugby players in response to a single game or season long competition. Significant transient and often sustained increases in inflammation, oxidative stress, muscle damage, psychophysiological stress, cardiovascular stress, monoamine neurotransmitter synthesis and suppression of the immune system were identified.

The selected markers were similarly combined to assess various physiological changes, work-load and performance aspects of professional rugby union. Impact induced skeletal muscle myoglobin release was found to contribute to oxidative stress *in vitro* and *in vivo*. Global positioning system technology was also found not be an accurate measure of cardiovascular work-load, whilst backs completed more total distance and carried the ball into contact more often than their forward counterparts who were involved in more total impacts; however no physiological difference was observed between positions. Furthermore, the selected biomarkers also showed that regardless of the “next-day” recovery strategy employed by a professional rugby team, players are still in the same psychophysiological state ~ 36 hours post-game if cold water immersion, compression, protein-carbohydrate nutrition and sleep are immediately completed post-game.

The final aspect improved the technically demanding and expensive urinary myoglobin ELISA assay. A reliable alternative RP-HPLC was developed that also improved sample stabilisation. It detected significant elevations following a MMA contest that also resulted in inflammatory and oxidative stress elevations. These were found to be attenuated through cold water immersion therapy that may impart its properties through suppression of blood derived T-cell activation in conjunction with vasoconstriction.

Too often research is presented in a group context, both in an exercise setting and clinical environment. While these studies identify the “normal” response of the population or treatment group, there are always outliers within a subject pool who respond differently. In a rugby context, variables including strength, power, muscle density and size, psychological well-being, physiological mechanisms for recovery and adaptation, fitness (aerobic and anaerobic capacities), and in-game variables including tackle force, angle of a tackle, game-time, number of events participated in, and position, all contribute to the psychophysiological stress response of an individual. The intra-individual response to stress is remarkably different also. Whilst subjective and qualitative measures may provide a similar “workload”, the stress response can be noticeably different.

For these reasons, the measurement of psychophysiological stress biomarkers of professional athletes for the monitoring of acute and chronic stress is imperative. It supersedes and provides added benefit over the traditional questionnaires about perceived soreness, fatigue and energy levels and the use of non-individualised workload measures that include global positioning system (GPS) and video-analysis technology. Respecting each individual athlete as their own separate entity is essential for managing them effectively. This will allow for optimisation for physiological and psychological adaptation, growth and recovery.

One of the key points stated before the commencement of this thesis, was the practicality of the findings and how they could be used in a professional team setting to optimise performance and success. From a coach’s perspective, a viewpoint based on skill, fitness, strength and conditioning and medical, the information gathered from this thesis has revealed an insight into the rigors of the game that were until now unknown or un-quantified in the New Zealand game on such a large scale. The coach’s involved in this research were pleasantly surprised by the results of the studies conducted which has subsequently “opened their eyes” to the necessity of managing each player individually.

## 14.2 STRENGTHS AND LIMITATIONS

The greatest strength of this research was the identification of several unique and non-overlapping psychophysiological aspects of stress in a non-invasive, stress free and non-disruptive manner. Often research has measured several indices of oxidative stress or



immune system parameter changes. Whilst they do provide a great perspective on a particular function following a treatment or exercise protocol, the markers often over-lap one another. This research specifically identified unique markers that encompassed a wide variety of “stress” to gauge a true understanding of rugby-induced trauma. The measurement of immune system function, inflammation, oxidative stress, flight or fight response, cardiovascular stress, muscle damage and psychophysiological stress casts a comprehensive net over the “stress” response.

Furthermore, this was all achieved in a stress free manner through non-invasive sample collection utilising urine and saliva only. With season long assessment key to understanding psychophysiological changes in an athlete, consistent venepuncture is an aspect that quite often prevents voluntary participation on a large and continuous scale. All participants happily obliged with the testing protocol that required no more than two minutes of their time at training or on match day. This is a vital aspect that requires careful consideration due to the pre-game “rituals” of professional athletes. By keeping stress to a minimum, participation is maintained without disrupting the focus of an athlete.

Interpretation of biochemical analysis was an essential part of this thesis. Due to the lack of research on the psychophysiological demands of rugby union and how to proceed with the resultant information, clear interpretation and development of specific group and individual reference values was imperative. The diversification of the research team helped clarify and understand the meaning of the results in an exercise and medical context. Careful clarification of their meaning was crucial for the relay of the results to the team. Whilst this study did not have a control group, this provided an added advantage with each individual player acting as their own control. The added benefit of this analysis neglects the effect of other individuals on a specific player. This allows for precise monitoring of “stress” in comparison to a specific or repeated event.

However certain difficulties arise when working with professional athletes. Like all professions, decision making can be controlled by a controlling body. This is no different in a rugby setting and unfortunately assessing “stress” in a manner that provides the best representation of a players health is not always made possible due to the rigorous training schedules and requirements of the professional rugby player. As a result of the specific training and playing schedule, sample collection could have been more precise and effective.

For the majority of the season, three measurements were taken: pre-game, post-game and ~ 36 hours post-game. For a comprehensive evaluation, collection three and five days post-game would have been ideal. Moreover sample collection from players was not always at a 100 % success rate. On game day, some players would forget to provide samples or refuse to provide them with the notion it would disrupt their pre-game “ritual”. Similarly, sample collection ~ 36 hours later was governed by participation in the gym, ability to pass urine, and the presence of an injury or un-related rugby task which prevented them partaking in the normal gym session where collection took place. Unfortunately such complications are difficult to avoid, however it is just a facet of real-world research that is unavoidable.

Limitations also existed around the practicality and usage of such novel individualised information and how best to implement their meaning on the management of players. Since this type of assessment is so original in its plan of use, the ability of all coaches to utilise the information is rather limited. Throughout the course of the research, information was supplied to coaches of the training staff in the hope of providing them important individualised information that cannot be extracted from their current technologies. Missing was the link and ability to transfer the information analysed in a way that could be used to change and adapt a player based on their psychophysiological state. Limitations arise when the staff of a professional team are not specialised in their understanding of the specific biochemical information relayed to them from biochemical specialists. Obviously an effective means of communicating the information in an easily digestible manner is required.

Furthermore, because this information is so specialised, professional teams might be more reluctant to trust the information and its meaning, and rely solely on what has been proven to work on a consistent basis (GPS and video-analysis). The main flaw with this type of research is its ability to improve team performance and win championships. Essentially each player is managed as a business asset and improving that assets ability to perform on a week by week basis is vital for success. In the end, this technology is there to provide a service to improve their chances of this success. It has been shown to observe players in states of potential overreaching and over-training, but its ability to increase the chances of winning requires further investigation.

Money and time constraint was another limitation of this research which is no doubt a major consideration for future use of this technology. The total cost of consumables for the project

was in excess of \$30,000. This was purely for the consumables of the project and did not include machinery over-heads or labour that constitutes the major part of the work. Additionally, time constraints play a major part in the delivery of information in a manner that can be utilised by coaches to manage their players. The use of the information by the coaches meant completing the analysis overnight to avoid a delay that is so imperative for player management. Length of assays and number of assays also contributed to the total delay of analysis; a point to consider for any future use of this technology that could easily be avoided with multiple personnel.

### **14.3 PRACTICAL APPLICATIONS AND FUTURE WORK**

The applications and future work from this thesis are limitless. Originally this type of research was derived from a clinical background. With the realisation that it is an informative “predictive” tool that has the potential to manage players following trauma that could be comparable to victims in the ICU, its future applications could reside in a similar setting. More specifically, managing the recovery of post-operative patients, assessing the degree of severity of an acute victim and whether further examination is required, or monitoring the treatment of specific drugs and the progression of illnesses and diseases can be conducted. This may be an informative tool that could be introduced into a hospital that has the ability in a non-invasive manner to more accurately predict or assess a patient.

It also provides a new possibility in the management of athlete health. If managed correctly, it can be a cost-effective and important instrument for improving performance. World Rugby, like other governing bodies, is always significantly concerned with player welfare. With this type of research available and its effective individualised player assessment, this technology could quite possibly integrate or even supersede current technology in professional rugby and abroad.

One area of rugby related stress that has not been focused on biochemically is concussion and brain injury. Rugby based sports have the highest incidence rates of concussion in any organised sport (Hinton-Bayre *et al.*, 2004; Jordan and Bailes 2000; Kemp *et al.*, 2008; Makdissi *et al.*, 2009; Tommasone and McLeod 2006). Concussion in sport is described as a complex pathophysiological process affecting the brain induced by a near instant transfer of

kinetic energy (McCrory *et al.*, 2005). There is an increasing concern for the safety of players involved in contact related sports and the long term effects of repeated head trauma and concussion incidents. The acute effects of concussion or head trauma can alter postural stability and neuropsychological function (Guskiewicz *et al.*, 2007), while chronic deleterious effects can result in deterioration of planning and memory capacity (Guskiewicz *et al.*, 2005; Iverson *et al.*, 2004; Lowenstein *et al.*, 1992; Matser *et al.*, 2001) as a result of hippocampal neuronal damage (Lowenstein *et al.*, 1992; Mouzon *et al.*, 2012; Shultz *et al.*, 2012; Slemmer *et al.*, 2002). The long term effects of head trauma have been studied in retired American football players with those suffering multiple concussions performing worse on neuropsychological tests than those with one or none (Collins *et al.*, 1999; Guskiewicz *et al.*, 2005). In addition, slower recovery has been observed with each successive concussion with an increased prevalence of further events with the more experienced (Guskiewicz *et al.*, 2003; Slobounov *et al.*, 2007). This may be a consequence of sports related concussions resulting in long-term motor system dysfunctions that seem attributable to subclinical intra-cortisol inhibitory system abnormalities with subsequent events exacerbating this deficit (De Beaumont *et al.*, 2007). In contrast however, there has been shown to be no cumulative effect of one or two previous concussions, nor are returning players at further risk of an injury (Iverson *et al.*, 2006; Pellman *et al.*, 2005).

Brain injury quantification and measurement is often performed through expensive and time consuming scans like magnetic resonance imaging (MRI) and computerised tomography (CT) (Gentry *et al.*, 1988; Wilde *et al.*, 2005), or through simple subjective neurological examinations by trained physicians. ELISA based technology of specific brain injury markers in sport has not been explored in great depth, but may provide a more cost-effective and reliable method of brain injury quantification. Invasive analysis through serum or cerebrospinal fluid (CSF) has been used extensively for quantification assessment of traumatic brain injury (TBI). As many as 99 biomarkers have been used to assess severity (Papa *et al.*, 2013), including S100 calcium binding protein B (S100B), neurofilament, glial fibrillary acidic protein (GFAP), ubiquitin carboxyl terminal hydrolase-L1 (UCH-L1), creatine kinase brain (CK-BB), myelin basic protein, micro RNA's (Redell *et al.*, 2010) and lactate dehydrogenase (Yokobori *et al.*, 2013).

Neuron specific enolase (NSE) may offer a non-invasive specific alternative. It is a 78 kDa glycolytic protein located primarily in the neuronal cytoplasm and dendrites (Finnoff *et al.*, 2011) which has been established as marker of brain damage following traumatic brain injury (Herrmann *et al.*, 2000; Raabe *et al.*, 1999). Unlike S100B which has been questioned about its specificity to head trauma and its change dependent on exercise intensity and duration (Anderson *et al.*, 2001; Banks 1999; de Oliveira Dietrich *et al.*, 2004; Dietrich *et al.*, 2003; Herrmann 2001; Kleine *et al.*, 2003; Koh and Lee 2013; Marchi *et al.*, 2004; Michetti *et al.*, 2011; Raabe 2001; Steiner *et al.*, 2011; Straume-Næsheim *et al.*, 2008; Undén *et al.*, 2005), NSE has been shown to have greater specificity in assessing brain injury with concentrations correlating with brain injury severity (Skogseid *et al.*, 1992). S100B does show promise however, with its link to non-invasive assessment in asphyxiated newborns through saliva analysis (Gazzolo *et al.*, 2015) and its much smaller 21 kDa molecular weight.

Even though NSE is present in tissues rich in smooth muscle cells (Haimoto *et al.*, 1985), it is at a concentration  $1/50^{\text{th}}$  of the brain suggesting injuries to organs will not increase serum concentrations (Kleine *et al.*, 2003). Moreover, it is not a marker of BBB integrity (Kapural *et al.*, 2002), it has been shown to rise in response to exercise induced head trauma (Graham *et al.*, 2011; Horner *et al.*, 1993), and is also a good prognostic marker of traumatic brain injury or cerebral complications (Beaudeau *et al.*, 2000; Herrmann *et al.*, 2000; Johnsson *et al.*, 1995; Marangos *et al.*, 1980; Zitnay *et al.*, 2008). Its 78 kDa size however makes it an ideal serum marker but questions its sensitivity in urine as a result of the glomerular pore radius. With minimal research regarding its concentration changes in urine to date (Gotoh *et al.*, 1986), high concentrations as a result of suspected brain injury might be a result of acute kidney damage which therefore require albumin correction.

To my knowledge, there has been no research that has quantitatively measured head trauma associated with rugby union using a biomarker. New concussion laws have been developed in rugby union that allows a player who may have received a concussive impact to be removed from the field and analysed before returning to play. Players who are concussed are put through rigorous screening tests before returning to play, however little evidence or knowledge is known about the level of brain injury sustained in contact sports of those players who do not suffer a concussion. With incidence rates higher than any other sport, it is crucial to have an increased understanding of the acute level of brain injury or trauma

following a single game of rugby union to realise the severity and therefore adapting and monitoring a player accordingly.

Neuron specific enolase may have the potential to identify recovery progression following a concussive or traumatic incident that can be used to inform players and management about when they are ready to return to training and playing. Future research in this specific area of non-invasive biochemical stress assessment should consider brain injury monitoring following the large increase in concussive incidents in today's modern game.

Additionally identifying differences between functional overreaching (FO), non-functional overreaching (NFO), and overtraining syndrome (OTS) can often be more qualitative than quantitative. A clear consensus exists on the definitions between these (Meeusen *et al.*, 2013), however in a team sport (37-man squad) where games are played on a weekly basis and the more skilled players are required or needed to play, identifying those suffering from any of these conditions can become problematic. Performance decrement in professional rugby is based on under-recovery, a result of excessively prolonged or intense exercise, stressful competition, or other stresses which lead to progressive fatigue and underperformance (Budgett 1998). This may be a result of ignoring the signs of fatigue, heavy muscles, and depression until performance is chronically affected (Dymont 1993), which in today's modern era of professional rugby is essential for player longevity and team performance. Cunniffe *et al.* (2011) monitored professional rugby players over a season and noticed a trend between the number of upper respiratory infections and salivary immunoglobulin A (sIgA) concentrations which is similar to that observed in elite swimmers (Gleeson *et al.*, 1999). Thought to occur via certain stressors of physiological, psychological and environmental nature, professional rugby has been identified as a sport capable of inducing FO, NFO or OTS.

Several markers including blood lactate, nor-adrenaline and maximal heart rate have been postulated as reliable indicators of FO, NFO and OTS (Lehmann *et al.*, 1992). Biochemical markers of immune status have also been regularly used to predict athletes in a state of FO/NFO/OTS (Gleeson *et al.*, 1999). Future work could utilise the effectiveness of these markers in quantifying physiological stress and compare it to changes in traditional performance measures and questionnaires about perceived soreness and fatigue.

# Chapter 15

## References

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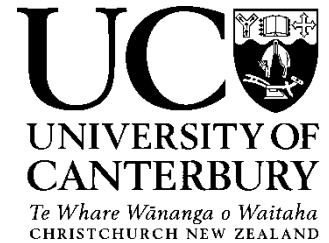
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# Chapter 16

## APPENDICES

## APPENDIX A



*Angus Lindsay  
School of Biological Sciences  
University of Canterbury  
Private Bag 4800  
Christchurch  
8140*

*June 4<sup>th</sup> 2013*

### CONSENT FORM

#### Urinary neopterin in competitive bodybuilders

I have read and understood the description of the above-named project. On this basis I agree to participate as a subject in the project, and I consent to publication of the results of the project with the understanding that anonymity will be preserved.

I understand that comments I make may be written down and used in the report discussion.

I understand that my name will not be written down next to my comments and that my name will not be used in any part of the results, data, and final report.

I understand also that I may withdraw at anytime from the project before data analysis has begun, including withdrawal of any information I have provided.

I note that the project has been reviewed *and approved* by the University of Canterbury Human Ethics Committee.

NAME (please print): .....

Signature:

Date:

- 
1. This project has been reviewed and approved by the University of Canterbury Human Ethics Committee low risk process
  2. Complaints may be addressed to:  
The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

## APPENDIX B

Angus Lindsay  
School of Biological Sciences  
University of Canterbury  
Private Bag 4800  
Christchurch  
8140

June 4<sup>th</sup> 2013



### INFORMATION

You are invited to participate as a subject in the research project, Urinary neopterin in competitive bodybuilders.

The aim of this project is to analyse the concentration of neopterin (NP) and 7,8-dihydroneopterin (78NP), indicators of inflammation, immune system activation and oxidative stress, in competitive bodybuilders.

Your involvement in this project will be to provide urine samples, and the right to withdraw from the project at any time before data analysis has begun, including withdrawal of any information provided. The exact nature of the tests is described below:

You will be required to provide these samples once per day for seven days (Monday-Sunday). Urine samples will be collected through the use of a 60ml labeled pottle where you will be required to provide a specimen mid-stream each morning. All samples will require you to freeze them immediately and at the conclusion of the week, all samples will be collected by the lead investigator and transported to the University of Canterbury. All samples will be destroyed through autoclaving and buried at the conclusion of the study.

The information gathered from the sampling will provide the level of inflammation, immune system activation and oxidative stress encountered by a competitive body builder during a week of training.

There are some guidelines to be noted:

- Keep your training, eating, supplementation and recovery as per normal.

There is no risk to your well-being as part of this study. The information gathered will remain confidential between the testers and yourself and is necessary to determine any risks during testing.

- 
1. This project has been reviewed and approved by the University of Canterbury Human Ethics Committee low risk process
  2. Complaints may be addressed to:  
The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

## APPENDIX B



As a participant, you can request a copy of any publication of this research. You will also be told any new information about adverse or beneficial effects related to the study that becomes available which may have an impact on your health.

The information gathered will remain confidential between the research team and yourself. In addition, you will be provided with a summary of your results and an interpretation of the data

The results of the project may be published, but you may be assured of the complete confidentiality of data gathered in this investigation. The identity of participants will not be made public. To ensure anonymity and confidentiality all data will be kept on the project leader's personal computer in a locked office.

The project is being carried out by Angus Lindsay, Steven Gieseg and Nick Draper, who can be contacted at [angus.lindsay@pg.canterbury.ac.nz](mailto:angus.lindsay@pg.canterbury.ac.nz), [steven.gieseg@canterbury.ac.nz](mailto:steven.gieseg@canterbury.ac.nz), [nick.draper@canterbury.ac.nz](mailto:nick.draper@canterbury.ac.nz), respectively. They will be pleased to discuss any concerns you may have about participation in the project. Your individual results can be discussed with you at the end of the week if you so desire, and a summary of the results will be offered to you as a participant at the end of the study.

- 
1. This project has been reviewed and approved by the University of Canterbury Human Ethics Committee low risk process
  2. Complaints may be addressed to:  
The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

## APPENDIX C



*Angus Lindsay  
School of Biological Sciences  
University of Canterbury  
Private Bag 4800  
Christchurch  
8140*

*June 4<sup>th</sup> 2013*

### INFORMATION

You are invited to participate as a subject in the research project, Urinary neopterin in competitive bodybuilders.

The aim of this project is to analyse the concentration of neopterin (NP) and 7,8-dihydroneopterin (78NP), indicators of inflammation, immune system activation and oxidative stress, in competitive bodybuilders.

Your involvement in this project will be to provide urine samples, and the right to withdraw from the project at any time before data analysis has begun, including withdrawal of any information provided. The exact nature of the tests is described below:

You will be required to provide a sample for use as a control. Urine samples will be collected through the use of a 60ml labeled pottle where you will be required to provide a specimen mid-stream. All samples will be destroyed through autoclaving and buried at the conclusion of the study.

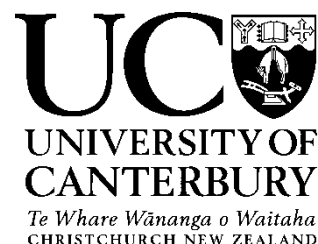
The information gathered from the sampling will provide the level of inflammation, immune system activation and oxidative stress compared to a competitive body builder during a week of training.

There is no risk to your well-being as part of this study. The information gathered will remain confidential between the testers and yourself and is necessary to determine any risks during testing.

As a participant, you can request a copy of any publication of this research. You will also be told any new information about adverse or beneficial effects related to the study that becomes available which may have an impact on your health.

- 
1. This project has been reviewed and approved by the University of Canterbury Human Ethics Committee low risk process
  2. Complaints may be addressed to:  
The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

## APPENDIX C



The information gathered will remain confidential between the research team and yourself. In addition, you will be provided with a summary of your results and an interpretation of the data.

The results of the project may be published, but you may be assured of the complete confidentiality of data gathered in this investigation. The identity of participants will not be made public. To ensure anonymity and confidentiality all data will be kept on the project leader's personal computer in a locked office.

The project is being carried out by Angus Lindsay, Steven Gieseg and Nick Draper, who can be contacted at [angus.lindsay@pg.canterbury.ac.nz](mailto:angus.lindsay@pg.canterbury.ac.nz), [steven.gieseg@canterbury.ac.nz](mailto:steven.gieseg@canterbury.ac.nz), [nick.draper@canterbury.ac.nz](mailto:nick.draper@canterbury.ac.nz), respectively. They will be pleased to discuss any concerns you may

have about participation in the project. Your individual results can be discussed with you at the end of the week if you so desire, and a summary of the results will be offered to you as a participant at the end of the study.

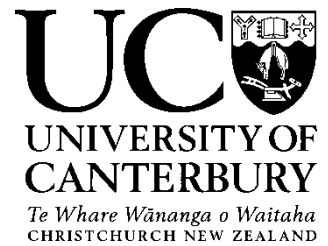
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  2. Complaints may be addressed to:  
The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)



## APPENDIX D

Free Radical Biochemistry Laboratory  
School of Biological Sciences  
College of Science

Tel: +64 3 364 2500, FAX: + 64 3 364 2590, [www.biol.canterbury.ac.nz](http://www.biol.canterbury.ac.nz)



# Participant Questionnaire

Name \_\_\_\_\_

Email address \_\_\_\_\_

Sex (circle one)      Male      Female

Date of birth \_\_\_\_\_

Weight (kg) \_\_\_\_\_

Height (cm) \_\_\_\_\_

Health Status \_\_\_\_\_

How are you feeling? \_\_\_\_\_

\_\_\_\_\_

What medication have you taken in the last 7 days (including pain killers)? \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

1. This project has been reviewed and approved by the University of Canterbury Human Ethics Committee low risk process
2. Complaints may be addressed to:  
The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

## APPENDIX D



If you are taking a dietary supplement, what are you taking? \_\_\_\_\_

\_\_\_\_\_

What are your bodybuilding credentials? \_\_\_\_\_

\_\_\_\_\_

How many calories a day do you consume? \_\_\_\_\_

What cycle of training are you in? \_\_\_\_\_

How many days a week do you train? \_\_\_\_\_

How many hours sleep do you get each night? \_\_\_\_\_

Health status 72 hours post final sample collection \_\_\_\_\_

\_\_\_\_\_

- 
1. This project has been reviewed and approved by the University of Canterbury Human Ethics Committee low risk process
  2. Complaints may be addressed to:  
The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

## APPENDIX E

Free Radical Biochemistry Laboratory  
School of Biological Sciences  
College of Science

Tel: +64 3 364 2500, FAX: + 64 3 364 2590, [www.biol.canterbury.ac.nz](http://www.biol.canterbury.ac.nz)



# Questionnaire for healthy volunteers

Name \_\_\_\_\_

Email address \_\_\_\_\_

Sex (circle one)      Male      Female

Date of birth \_\_\_\_\_

Weight (kg) \_\_\_\_\_

Height (cm) \_\_\_\_\_

Health Status \_\_\_\_\_

\_\_\_\_\_

How are you feeling? \_\_\_\_\_

\_\_\_\_\_

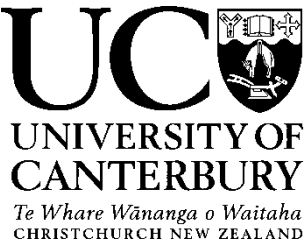
What medication have you taken in the last 7 days (including pain killers)? \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

1. This project has been reviewed and approved by the University of Canterbury Human Ethics Committee low risk process
2. Complaints may be addressed to:  
The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

# APPENDIX E



If you are taking a dietary supplement, what are you taking? \_\_\_\_\_  
\_\_\_\_\_

How would you describe your activity level? (circle one)

Low                                      Moderate                                      High

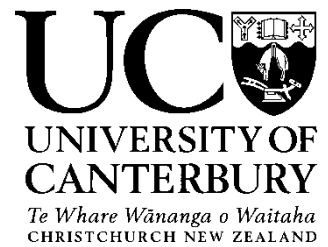
Physical activity in past 48 hours \_\_\_\_\_  
\_\_\_\_\_

Health status 72 hours post sample collection \_\_\_\_\_  
\_\_\_\_\_

- 
1. This project has been reviewed and approved by the University of Canterbury Human Ethics Committee low risk process
  2. Complaints may be addressed to:  
The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

## APPENDIX F

*Angus Lindsay  
School of Biological Sciences  
University of Canterbury  
Private Bag 4800  
Christchurch  
8140  
June 22<sup>nd</sup> 2013*



### CONSENT FORM

#### **The effect of acute and chronic muscle damage, inflammation, psychophysiological stress and immune function on player recovery in professional rugby**

I have read and understood the description of the above-named project. On this basis I agree to participate as a subject in the project, and I consent to publication of the results of the project with the understanding that anonymity will be preserved.

I understand that comments I make may be written down and used in the report discussion.

I understand that my name will not be written down next to my comments and that my name will not be used in any part of the results, data, and final report.

I understand also that I may at any time withdraw from the project before analysis has begun, including withdrawal of any information I have provided.

I understand the researchers will store a specimen of my urine and saliva for its later use as a part of this study.

I consent to any remaining samples being disposed of using standard disposal methods at the end of the study. YES/NO

I consent to any remaining samples being disposed of at the end of the study (please tick one):

Using standard disposal methods ☐

Disposed with appropriate karakia ☐

- 
1. This project has been reviewed and approved by the University of Canterbury Human Ethics Committee low risk process
  2. Complaints may be addressed to:  
The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

APPENDIX F



I wish to have my donated body part returned to me at the end of the study  
YES/NO

I note that the project has been reviewed *and approved* by the University of Canterbury Human Ethics Committee.

NAME (please print): .....

Signature:

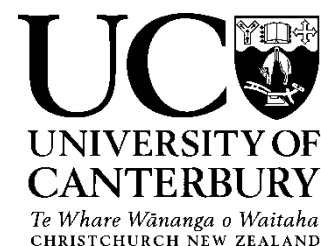
Date:

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1. This project has been reviewed and approved by the University of Canterbury Human Ethics Committee low risk process  
2. Complaints may be addressed to:  
The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

## APPENDIX G

Free Radical Biochemistry Laboratory  
School of Biological Sciences  
College of Science  
Tel: +64 3 364 2500, FAX: + 64 3 364 2590, [www.biol.canterbury.ac.nz](http://www.biol.canterbury.ac.nz)  
Email: [angus.lindsay@pg.canterbury.ac.nz](mailto:angus.lindsay@pg.canterbury.ac.nz)



### INFORMATION SHEET

**TITLE:** The effect of acute and chronic stress on muscle damage, inflammation and psychophysiological stress on player recovery in professional rugby

#### PRINCIPAL INVESTIGATOR:

Angus Lindsay, Free Radical Biochemistry Laboratory, School of Biological Sciences, University of Canterbury, Christchurch, New Zealand.

#### ABOUT THE STUDY

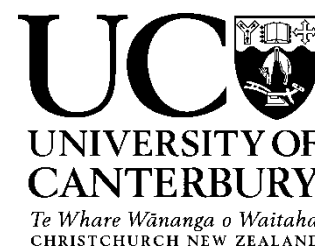
The purpose of this study is to identify the level of “stress” elicited by a single game and season of professional rugby union on player recovery. Little information is available using biochemical data and at the conclusion of this study, scientific data will illustrate what sort of stress accompanies a game. The information gathered from sampling will provide us with the individual response of each player to the acute and chronic stress of professional rugby. This will enable us to observe accurately the correlation between position and stress, as well as the recovery ability of each individual.

#### PARTICIPATION

Your participation is **entirely voluntary** (your choice). We will require you to state that you are healthy by filling in a questionnaire. You do not have to take part in this study. If you do agree to take part you are free to withdraw from the study at any time before data analysis has begun, including the withdrawal of any information without having to give a reason.

- 
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  2. Complaints may be addressed to:  
The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

## APPENDIX G



You will be required to provide urine and saliva samples to assess baseline levels during a week of easy training, and at five stages during a selected home game during the 2013 ITM Cup season. Biological specimen collection will occur at the team location on a given day. Saliva samples will be collected using a 50ml tube. This includes salivating into the tube for one minute. Urine samples will be collected using a 60ml pottle with a mid-stream sample required.

### RISKS AND BENEFITS

**There are no risks involved in this study, however if you have any issues or problems after the sample collections contact Angus Lindsay (027 320 5373) as soon as possible**

### COMPENSATION

In the unlikely event of a physical injury as a result of your participation in this study, you may be covered by ACC under the Injury Prevention, Rehabilitation and Compensation Act. ACC cover is not automatic and your case will need to be assessed by ACC according to the provisions of the 2002 Injury Prevention Rehabilitation and Compensation Act. If your claim is accepted by ACC, you still might not get any compensation. This depends on a number of factors such as whether you are an earner or non-earner. ACC usually provides only partial reimbursement of costs and expenses and there may be no lump sum compensation payable. There is no cover for mental injury unless it is a result of physical injury. If you have ACC cover, generally this will affect your right to sue the investigators. If you have any questions about ACC, contact your nearest ACC office or the investigator.

### GENERAL

If you have any questions about this research please phone Angus Lindsay on 0273205373. If you have any queries or concerns regarding your rights as a participant in this study, you may wish to contact an independent Health and Disability Advocate, as follows:

South Island 0800 377 766

Free Fax (NZ wide) 0800 2787 7678 (0800 2 SUPPORT)

Email (NZ wide) [advocacy@hdc.org.nz](mailto:advocacy@hdc.org.nz)

- 
1. This project has been reviewed and approved by the University of Canterbury Human Ethics Committee low risk process
  2. Complaints may be addressed to:  
The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)



## APPENDIX H

Free Radical Biochemistry Laboratory  
School of Biological Sciences  
College of Science

Tel: +64 3 364 2500, FAX: + 64 3 364 2590, [www.biol.canterbury.ac.nz](http://www.biol.canterbury.ac.nz)



### Player Questionnaire

Name\_\_\_\_\_

Height (cm)\_\_\_\_\_

Weight (kg)\_\_\_\_\_

Age\_\_\_\_\_

Game day nutrition\_\_\_\_\_

Game day recovery strategy\_\_\_\_\_

Game day sleep\_\_\_\_\_

Post-game day nutrition\_\_\_\_\_

\_\_\_\_\_

Post-game day hours of sleep\_\_\_\_\_

Post-game day activities\_\_\_\_\_

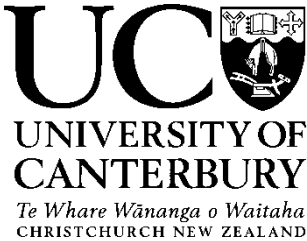
48 hours nutrition\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

1. This project has been reviewed and approved by the University of Canterbury Human Ethics Committee low risk process
2. Complaints may be addressed to:  
The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

## APPENDIX H



48 hours sleep\_\_\_\_\_

48 hours activities\_\_\_\_\_

72 hours nutrition\_\_\_\_\_

72 hours sleep\_\_\_\_\_

72 hours activities\_\_\_\_\_

96 hours nutrition\_\_\_\_\_

96 hours sleep\_\_\_\_\_

96 hours activities\_\_\_\_\_

120 hours nutrition\_\_\_\_\_

120 hours sleep\_\_\_\_\_

120 hours activities\_\_\_\_\_

- 
1. This project has been reviewed and approved by the University of Canterbury Human Ethics Committee low risk process
  2. Complaints may be addressed to:  
The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

## APPENDIX I

Free Radical Biochemistry Laboratory

School of Biological Sciences

College of Science

Tel: +64 3 364 2500, FAX: + 64 3 364 2590, [www.biol.canterbury.ac.nz](http://www.biol.canterbury.ac.nz)

## Health Screen for participants



Please complete the following questionnaire to assess your past and current health. If you answer YES to any question, please describe briefly if you wish.

## 1. At present, do you have any health problems for which you are:

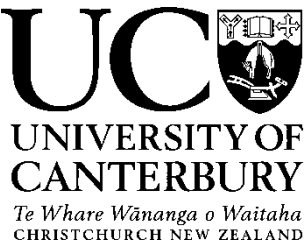
- |   |     |                          |    |                          |
|---|-----|--------------------------|----|--------------------------|
| a. On medication, prescribed or otherwise | Yes | <input type="checkbox"/> | No | <input type="checkbox"/> |
| b. Attending your general practitioner    | Yes | <input type="checkbox"/> | No | <input type="checkbox"/> |
| c. On a hospital waiting list             | Yes | <input type="checkbox"/> | No | <input type="checkbox"/> |

## 2. Have you ever had, or presently suffer from the following:

- |  |     |                          |    |                          |
|--|-----|--------------------------|----|--------------------------|
| a. Convulsions/epilepsy.....                               | Yes | <input type="checkbox"/> | No | <input type="checkbox"/> |
| b. Asthma.....   | Yes | <input type="checkbox"/> | No | <input type="checkbox"/> |
| c. Does your chest <i>tighten up</i> during exercise?..... | Yes | <input type="checkbox"/> | No | <input type="checkbox"/> |
| d. Diabetes.....   | Yes | <input type="checkbox"/> | No | <input type="checkbox"/> |
| e. A blood disorder.....                                   | Yes | <input type="checkbox"/> | No | <input type="checkbox"/> |
| f. Arthritis.....  | Yes | <input type="checkbox"/> | No | <input type="checkbox"/> |
| g. Digestive problems.....                                 | Yes | <input type="checkbox"/> | No | <input type="checkbox"/> |
| h. Heart problems.....                                     | Yes | <input type="checkbox"/> | No | <input type="checkbox"/> |
| i. Thyroid problems.....                                   | Yes | <input type="checkbox"/> | No | <input type="checkbox"/> |
| j. Post viral fatigue.....                                 | Yes | <input type="checkbox"/> | No | <input type="checkbox"/> |
| k. Flu in last 3 months.....                               | Yes | <input type="checkbox"/> | No | <input type="checkbox"/> |
| l. Cold in last 4 weeks.....                               | Yes | <input type="checkbox"/> | No | <input type="checkbox"/> |
| m. Kidney or liver problems.....                           | Yes | <input type="checkbox"/> | No | <input type="checkbox"/> |
| n. History of blood clots/poor circulation.....            | Yes | <input type="checkbox"/> | No | <input type="checkbox"/> |
| o. Known allergies.....                                    | Yes | <input type="checkbox"/> | No | <input type="checkbox"/> |

- 
- This project has been reviewed and approved by the University of Canterbury Human Ethics Committee low risk process
  - Complaints may be addressed to:  
The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

APPENDIX I



3. Has any, otherwise healthy, member of your family under the age of 35 died suddenly during or soon after exercise?..... Yes ☐ No ☐
4. Are you presently taking any antibiotics?..... Yes ☐ No ☐

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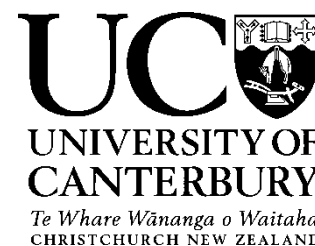
1. This project has been reviewed and approved by the University of Canterbury Human Ethics Committee low risk process

2. Complaints may be addressed to:  
The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

## APPENDIX J

Angus Lindsay  
School of Biological Sciences  
University of Canterbury  
Private Bag 4800  
Christchurch  
8140

September 5<sup>th</sup> 2013



### CONSENT FORM

#### **The effect of acute and chronic muscle damage, inflammation, psychophysiological stress and immune function on player recovery in professional rugby**

I have read and understood the description of the above-named project. On this basis I agree to participate as a subject in the project, and I consent to publication of the results of the project with the understanding that anonymity will be preserved.

I understand that comments I make may be written down and used in the report discussion.

I understand that my name will not be written down next to my comments and that my name will not be used in any part of the results, data, and final report.

I understand also that I may at any time withdraw from the project before analysis has begun, including withdrawal of any information I have provided.

I understand the researchers will store a specimen of my urine and saliva for its later use as a part of this study.

I consent to any remaining samples being disposed of using standard disposal methods at the end of the study. If you would like your samples disposed of using appropriate karakia please advise.

I note that the project has been reviewed *and approved* by the University of Canterbury Human Ethics Committee.

- 
1. This project has been reviewed and approved by the University of Canterbury Human Ethics Committee low risk process
  2. Complaints may be addressed to:  
The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

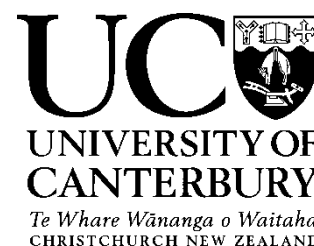
## APPENDIX J

[illegible]

1. This project has been reviewed and approved by the University of Canterbury Human Ethics Committee low risk process
2. Complaints may be addressed to:  
The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

## APPENDIX K

Free Radical Biochemistry Laboratory  
School of Biological Sciences  
College of Science  
Tel: +64 3 364 2500, FAX: + 64 3 364 2590, [www.biol.canterbury.ac.nz](http://www.biol.canterbury.ac.nz)  
Email: [angus.lindsay@pg.canterbury.ac.nz](mailto:angus.lindsay@pg.canterbury.ac.nz)



### INFORMATION SHEET

**TITLE:** The effect of acute and chronic stress on muscle damage, inflammation and psychophysiological stress on player recovery in professional rugby

#### PRINCIPAL INVESTIGATOR:

Angus Lindsay, Free Radical Biochemistry Laboratory, School of Biological Sciences, University of Canterbury, Christchurch, New Zealand.

#### ABOUT THE STUDY

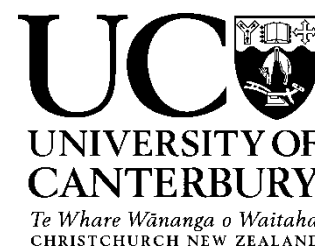
The purpose of this study is to identify the level of “stress” elicited by a single game and season of professional rugby union on player recovery. Little information is available using biochemical data and at the conclusion of this study, scientific data will illustrate what sort of stress accompanies a game. The information gathered from sampling will provide us with the individual response of each player to the acute and chronic stress of professional rugby. This will enable us to observe accurately the correlation between position and stress, as well as the recovery ability of each individual.

#### PARTICIPATION

Your participation is **entirely voluntary** (your choice). We will require you to state that you are healthy by filling in a questionnaire. You do not have to take part in this study. If you do agree to take part you are free to withdraw from the study at any time before data analysis has begun, including the withdrawal of any information without having to give a reason.

- 
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The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

## APPENDIX K



You will be required to provide urine and saliva samples to assess baseline levels during a week of easy training, and at five stages during a selected home game during the 2013 ITM Cup season. Biological specimen collection will occur at the team location on a given day.

Saliva samples will be collected using a 50ml tube. This includes salivating into the tube for one minute. Urine samples will be collected using a 60ml pottle with a mid-stream sample required.

### RISKS AND BENEFITS

**There are no risks involved in this study, however if you have any issues or problems after the sample collections contact Angus Lindsay (027 320 5373) as soon as possible**

### COMPENSATION

In the unlikely event of a physical injury as a result of your participation in this study, you may be covered by ACC under the Injury Prevention, Rehabilitation and Compensation Act. ACC cover is not automatic and your case will need to be assessed by ACC according to the provisions of the 2002 Injury Prevention Rehabilitation and Compensation Act. If your claim is accepted by ACC, you still might not get any compensation. This depends on a number of factors such as whether you are an earner or non-earner. ACC usually provides only partial reimbursement of costs and expenses and there may be no lump sum compensation payable. There is no cover for mental injury unless it is a result of physical injury. If you have ACC cover, generally this will affect your right to sue the investigators. If you have any questions about ACC, contact your nearest ACC office or the investigator.

### GENERAL

If you have any questions about this research please phone Angus Lindsay on 0273205373. If you have any queries or concerns regarding your rights as a participant in this study, you may wish to contact an independent Health and Disability Advocate, as follows:

South Island 0800 377 766

Free Fax (NZ wide) 0800 2787 7678 (0800 2 SUPPORT)

Email (NZ wide) [advocacy@hdc.org.nz](mailto:advocacy@hdc.org.nz)

- 
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The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)



## APPENDIX K



### CONFIDENTIALITY

No material which could personally identify you will be used in any reports on this study. No label will be placed on your samples which identifies it as coming from you. Urine and saliva samples will be labelled only with the date of donation.

### RESULTS

The findings from this research will be published in the appropriate peer reviewed journals and will be presented at suitable national and international meetings. Some of the results will also be presented as parts of University of Canterbury research theses. You may also request your individual results at the end of the study.

### DISPOSAL OF SAMPLES

All samples will be disposed of using autoclaving in accordance with NZS 4304:2002

'Healthcare Waste Management'. There are two methods of disposal and you may choose to have your remaining samples disposed of using standard disposal methods or disposed of with appropriate karakia, at the end of the study. You also have the option of having your sample returned at the end of the study.

### STATEMENT OF APPROVAL

This project has been reviewed and approved by the University of Canterbury Human Ethics Committee.

**Please feel free to contact Angus Lindsay if you have any questions about this study.**

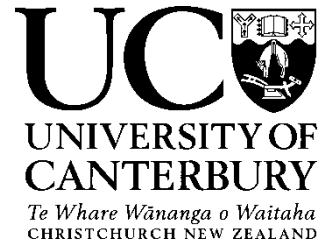
**Phone 0273205373, Email [angus.lindsay@pg.canterbury.ac.nz](mailto:angus.lindsay@pg.canterbury.ac.nz)**

- 
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The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

## APPENDIX L

Angus Lindsay  
School of Biological Sciences  
University of Canterbury  
Private Bag 4800  
Christchurch  
8140

October 29<sup>th</sup> 2013



### CONSENT FORM

#### **The effect of acute and chronic muscle damage, inflammation, psychophysiological stress and immune function on player recovery in professional rugby: phase 2**

I have read and understood the description of the above-named project. On this basis I agree to participate as a subject in the project, and I consent to publication of the results of the project with the understanding that anonymity will be preserved.

I understand that comments I make may be written down and used in the report discussion.

I understand that my name will not be written down next to my comments and that my name will not be used in any part of the results, data, and final report and I may request a summary of results.

I understand also that I may at any time withdraw from the project before analysis has begun, including withdrawal of any information I have provided.

I understand the results of the research will be seen by the research team as well as coaching, medical and training staff of the Canterbury Crusaders.

I understand the containers used for sample collection will have my initials on it for analysis identification purposes only and that it will only be seen by members of the research team.

I consent to any remaining samples being disposed of at the end of the study (please tick one):

Using standard disposal methods ☐

Disposed with appropriate karakia ☐

- 
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  2. Complaints may be addressed to:  
The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

# APPENDIX L



I wish to have my donated body part returned to me at the end of the study  
YES/NO

I note that the project has been reviewed *and approved* by the University of Canterbury Human Ethics Committee.

NAME (please print): .....

Signature:

Date:

---

1. This project has been reviewed and approved by the University of Canterbury Human Ethics Committee low risk process  
2. Complaints may be addressed to:  
The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

## APPENDIX M



Free Radical Biochemistry Laboratory  
School of Biological Sciences  
College of Science  
Tel: +64 3 364 2500, FAX: + 64 3 364 2590, [www.biol.canterbury.ac.nz](http://www.biol.canterbury.ac.nz)  
Email: [angus.lindsay@pg.canterbury.ac.nz](mailto:angus.lindsay@pg.canterbury.ac.nz)

### INFORMATION SHEET

**TITLE:** The effect of acute and chronic stress on muscle damage, inflammation and psychophysiological stress on player recovery in professional rugby

#### PRINCIPAL INVESTIGATOR:

Angus Lindsay, Free Radical Biochemistry Laboratory, School of Biological Sciences, University of Canterbury, Christchurch, New Zealand.

#### ABOUT THE STUDY

The purpose of this study is to identify the level of “stress” elicited by a single game and season of professional rugby union on player recovery. Little information is available using biochemical data and at the conclusion of this study, scientific data will illustrate what sort of stress accompanies a game. The information gathered from sampling will provide us with the individual response of each player to the acute and chronic stress of professional rugby. This will enable us to observe accurately the correlation between position and stress, as well as the recovery ability of each individual.

#### PARTICIPATION

Your participation is **entirely voluntary** (your choice). We will require you to state that you are healthy by filling in a questionnaire. You do not have to take part in this study. If you do

agree to take part you are free to withdraw from the study at any time before data analysis has begun, including the withdrawal of any information without having to give a reason.

You will be required to provide urine and saliva samples to assess baseline levels during a week of easy training, and at five stages during a selected home game during the 2013 ITM Cup season. Biological specimen collection will occur at the team location on a given day.

- 
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The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

## APPENDIX M



Saliva samples will be collected using a 50ml tube. This includes salivating into the tube for one minute. Urine samples will be collected using a 60ml pottle with a mid-stream sample required.

### RISKS AND BENEFITS

**There are no risks involved in this study, however if you have any issues or problems after the sample collections contact Angus Lindsay (027 320 5373) as soon as possible.**

### COMPENSATION

See following page.

### GENERAL

If you have any questions about this research please phone Angus Lindsay on 0273205373. If you have any queries or concerns regarding your rights as a participant in this study, you may wish to contact an independent Health and Disability Advocate, as follows:

South Island 0800 377 766

Free Fax (NZ wide) 0800 2787 7678 (0800 2 SUPPORT)

Email (NZ wide) [advocacy@hdc.org.nz](mailto:advocacy@hdc.org.nz)

### CONFIDENTIALITY

No material which could personally identify you will be used in any reports on this study. Urine and saliva samples will be labelled with your initials which identifies it as coming from you which is strictly for analysis identification and will only be accessible by members of the research team.

Results will be seen by the research team as well as coaching, medical and training staff of the Canterbury Crusaders.

### RESULTS

The findings from this research will be published in the appropriate peer reviewed journals and will be presented at suitable national and international meetings. Some of the results will also be presented as parts of University of Canterbury research theses. You may also request your individual results at the end of the study. Results from the

- 
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The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

## APPENDIX M

research will be closely monitored by coaching and training staff to observe the level of “stress” of each player throughout the season but will not be used to alter any recovery or training strategies for the 2014 season.

### **DISPOSAL OF SAMPLES**

All samples will be disposed of using autoclaving in accordance with NZS 4304:2002 'Healthcare Waste Management'. There are two methods of disposal and you may choose to have your remaining samples disposed of using standard disposal methods or disposed of with appropriate karakia, at the end of the study. You also have the option of having your sample returned at the end of the study. Samples will be destroyed once analysed and the raw data will be kept for a period of 10 years.

- 
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The Chair  
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## APPENDIX M



4 December 2013

Player

C/- Super Rugby Franchise

Dear Player

### IMPORTANT EMPLOYEE INFORMATION—ACC CLAIMS & ENTITLEMENTS

You may be aware that the NZRU has been an Accredited Employer through the ACC Partnership Programme since 2000. Essentially this means that the NZRU directly manages and pays for all of its own work-related employee accidents and injuries.

The scheme also ensures that you are provided with access to the most timely and appropriate medical treatment and rehabilitation programmes available. By managing your injury needs in-house we can ensure you are managed as an individual and not as part of a wider ACC group of claimants.

As part of our membership in the ACC Partnership Programme, we are required to annually provide all employees information about how to access the programme and the key issues you need to be aware of.

**You should already have a black and silver individual employee information card** that includes details of the ACC Accredited Employer Programme procedures. **Please carry this card in your wallet and present it** each time you seek treatment for an injury from anyone other than your Team Doctor or Physiotherapist, so we can ensure that the correct information is recorded and also ensure the NZRU is billed on your behalf.

On the back of this card are contact details for the NZRU's Employee Assistance Programme providers, INSTEP Ltd, including the 24-hour access line for counselling support which you, or your family members, may access at any time. INSTEP is contracted to provide confidential support to our players and their families. Your PDM can provide more information if you are interested, and information brochures are available from your PDM, or the Contracts Managers in Professional Rugby at the NZRU.

The back of your employee information card also includes a couple of NZRU phone numbers that are important for you – payroll and contract enquiries.

**Enclosed with this letter is Professional Rugby Fact Sheet (ACC 001)**, which outlines summary information about the Accredited Employer Programme and your rights as an employee. You may wish to keep the fact sheet handy as a reference guide in the event that you are injured at work.

- 
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The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

## APPENDIX M



As most of your work related injuries will occur in a team environment, your Team Doctor, Coach and/or Manager will ensure you are guided through the process.

You should be aware of the following:

1. If you require treatment for a work-related injury, you should always use the specified NZRU-approved provider (in the first instance, Team Doctor or Physiotherapist).
2. If for any reason you need treatment or assessment from a health provider other than your Team Doctor or Physiotherapist, please ask them to send the invoice for their costs and all paperwork directly to **NZRU Medical Team, PO Box 2172, Wellington**, and not to ACC. Present them with your Accredited Employer Card to ensure correct billing.

Please do not hesitate to contact your Team Doctor or a member of the Professional Rugby Team if you have any questions or if you would like to discuss ACC or health and safety issues in more detail.

Yours sincerely

Neil Sorensen

**Professional Rugby Manager**

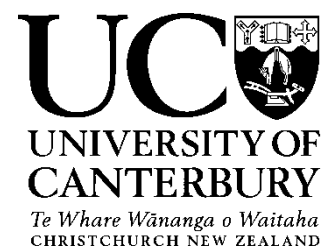


Principal Partner of New Zealand Rugby

- 
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The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)



## APPENDIX M



### RESEARCH TEAM

Angus Lindsay, Steven Gieseg, Nick Draper, Nic Gill, Mark Drury, Simon Thomas, Deb Robinson, Carl Scarrott, John Lewis, John Pickering and Geoff Shaw.

### STATEMENT OF APPROVAL

This project has been reviewed and approved by the University of Canterbury Human Ethics Committee.

Postal address: The Secretary  
Human Ethics Committee  
Okeover House  
Private Bag 4800  
University of Canterbury  
New Zealand

Email address: [human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

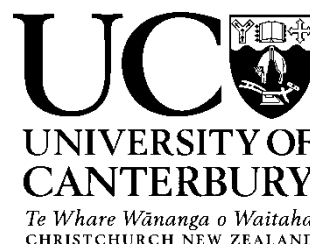
**Please feel free to contact Angus Lindsay if you have any questions about this study.**

**Phone 0273205373, Email [angus.lindsay@pg.canterbury.ac.nz](mailto:angus.lindsay@pg.canterbury.ac.nz)**

**You can also contact The Research Head for the Laboratory and Supervisor of the project A/Prof Steven Gieseg, Phone 03 364 2987 ext 7049 or email [steven.gieseg@canterbury.ac.nz](mailto:steven.gieseg@canterbury.ac.nz)**

- 
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The Chair  
[human-ethics@canterbury.ac.nz](mailto:human-ethics@canterbury.ac.nz)

## APPENDIX N



*Angus Lindsay  
School of Biological Sciences  
University of Canterbury  
Private Bag 4800  
Christchurch  
8140*

*3<sup>rd</sup> March 2015*

### CONSENT FORM

#### **Testing the effect of cold water immersion on physiological stress following a Mixed Martial Arts Contest.**

I have read and understood the description of the above-named project. On this basis I agree to participate as a subject in the project, and I consent to publication of the results of the project with the understanding that anonymity will be preserved.

I understand that comments I make may be written down and used in the report discussion.

I understand that my name will not be written down next to my comments and that my name will not be used in any part of the results, data, and final report.

I understand also that I may at any time withdraw from the project before analysis has begun, including withdrawal of any information I have provided.

I understand the researchers will store a specimen of my urine and saliva for its later use as a part of this study.

I consent to any remaining samples being disposed of using standard disposal methods at the end of the study.

YES/NO

APPENDIX N

I consent to any remaining samples being disposed of at the end of the study (please tick one):

Using standard disposal methods

☐

Disposed with appropriate karakia

☐

(If applicable)

I wish to have my donated urine sample returned to me at the end of the study      YES/NO

I note that the project has been reviewed *and approved* by the University of Canterbury Human Ethics Committee.

NAME (please print): .....

Signature:

Date:

## APPENDIX O

Free Radical Biochemistry Laboratory  
School of Biological Sciences  
College of Science  
Tel: +64 3 364 2500, FAX: + 64 3 364 2590, [www.biol.canterbury.ac.nz](http://www.biol.canterbury.ac.nz)  
Email: [angus.lindsay@pg.canterbury.ac.nz](mailto:angus.lindsay@pg.canterbury.ac.nz)



### INFORMATION SHEET

**TITLE:** Testing the effect of cold water immersion on physiological stress following a mixed martial arts contest

#### PRINCIPAL INVESTIGATOR:

Angus Lindsay, Free Radical Biochemistry Laboratory, School of Biological Sciences, University of Canterbury, Christchurch, New Zealand.

#### ABOUT THE STUDY

The purpose of this study is to identify the effect of cold water immersion in comparison to passive recovery on changes in physiological stress suffered in a mixed martial arts contest. Minor information is currently available on the stress associated with the sport and currently no data pertaining to the effect of cold water immersion following a contest. At the conclusion of this study, scientific data will illustrate the type and scope of physiological stress accompanying such an event. The information gathered from sampling will provide us with the individual response of each athlete to the acute stress of a mixed martial arts contest and how this can be used to improve recovery.

#### PARTICIPATION

Your participation is **entirely voluntary** (your choice). You do not have to take part in this study. If you do agree to take part you are free to withdraw from the study at any time before data analysis has begun, including the withdrawal of any information without having to give a reason.

## APPENDIX O

You will be required to provide urine samples to assess biomarkers (markers of physiological stress) prior to the contest, immediately after the contest and again 1 hour, 2 hours, 24 hours and 48 hours after the contest has ended. Urine sample collection will occur at the contest location.

Urine samples will be collected using a 70 mL pottle (mid-stream sample is required). Providing a urine sample is non-invasive, easy to do and will require no more than two minutes to complete.

You will also be asked to complete a questionnaire about your individual characteristics as well as your competitive record and recovery protocol completed.

### RISKS AND BENEFITS

**Aside from the potential injury associated with the contest themselves, there is a very unlikely scenario that a temperature sensor may malfunction. However if you have any issues or problems after the sample collections contact Angus Lindsay as soon as possible.**

### COMPENSATION

In the unlikely event of a physical injury as a result of your participation in this study, you may be covered by ACC under the Injury Prevention, Rehabilitation and Compensation Act. ACC cover is not automatic and your case will need to be assessed by ACC according to the provisions of the 2002 Injury Prevention Rehabilitation and Compensation Act. If your claim is accepted by ACC, you still might not get any compensation. This depends on a number of factors such as whether you are an earner or non-earner. ACC usually provides

only partial reimbursement of costs and expenses and there may be no lump sum compensation payable. There is no cover for mental injury unless it is a result of physical injury. If you have ACC cover, generally this will affect your right to sue the investigators. If you have any questions about ACC, contact your nearest ACC office or the investigator.

## APPENDIX O

### GENERAL

If you have any questions about this research please phone Angus Lindsay on 0273205373. If you have any queries or concerns regarding your rights as a participant in this study, you may wish to contact an independent Health and Disability Advocate, as follows:

South Island 0800 377 766

Free Fax (NZ wide) 0800 2787 7678 (0800 2 SUPPORT)

Email (NZ wide) [advocacy@hdc.org.nz](mailto:advocacy@hdc.org.nz)

### CONFIDENTIALITY

No material which could personally identify you will be used in any reports in this study. No label will be placed on your samples which identifies it as coming from you. Urine samples will be labelled only with the date of donation.

### RESULTS

The findings from this research will be published in the appropriate peer reviewed journals and will be presented at suitable national and international meetings. Some of the results will also be presented as parts of University of Canterbury research theses. You may also request your individual results at the end of the study.

### DISPOSAL OF SAMPLES

All samples will be disposed of using autoclaving in accordance with NZS 4304:2002

'Healthcare Waste Management'. There are two methods of disposal and you may choose to have your remaining samples disposed of using standard disposal methods or disposed of with appropriate karakia, at the end of the study. You also have the option of having your sample returned at the end of the study.

## **APPENDIX O**

### **STATEMENT OF APPROVAL**

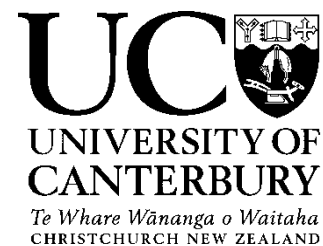
This project has been reviewed and approved by the University of Canterbury Human Ethics Committee.

**Please feel free to contact Angus Lindsay if you have any questions about this study.**

**Phone 0273205373, Email [angus.lindsay@pg.canterbury.ac.nz](mailto:angus.lindsay@pg.canterbury.ac.nz)**

## APPENDIX P

Free Radical Biochemistry Laboratory  
School of Biological Sciences  
College of Science  
Tel: +64 3 364 2500, FAX: + 64 3 364 2590, [www.biol.canterbury.ac.nz](http://www.biol.canterbury.ac.nz)



# Participant Questionnaire

Name\_\_\_\_\_

Height (cm)\_\_\_\_\_

Weight (kg)\_\_\_\_\_

Age\_\_\_\_\_

MMA record\_\_\_\_\_



APPENDIX Q

The addition of varying volumes of 0.2M NaOH on pH to 5 mL of urine from pre and post-game samples. This experiment was used to gauge the amount of buffer required to change the pH of pre and post-game urine samples from the professional studies to approximately 7 – 9.5. Changing the pH of the sample was conducted to stabilize any myoglobin present in the sample while also increasing the antibody binding properties for sensitivity and complete quantification.

